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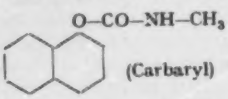
Pharmacological Effects of Carbaryl II. Modification of Serotonin Metabolism in the Rat Brain

In a previous paper[1] it was reported that the administration of a mixture of DDT, parathion and carbaryl (L-naphthyl-N-methylcarbamate) induced an increased elimination of urinary 3-methoxy-4-hydroxy-mandelic acid (VMA) and 5-hydroxy-3-indolylacetic acid (5-HIAA) in the rabbit. It was postulated that a stress mechanism is involved, featuring an increased synthesis rate of the catecholamines and Serotonin respectively. HASSAN[2] demonstrated an increased sympathoadrenergic activity in the rat, following the oral administration of carbaryl. This increased activity involved increased synthesis of norepinephrine periferally (and probably centrally) with concomitant increase of urinary VMA excretion.



(Carbaryl)

The purpose of the present investigation was to study the effect of a single carbaryl dose on serotonin metabolism in the rat brain.

Male albino rats (Holtzman) weighing 150–180 g were used in this study. All animals were housed in groups for several weeks, and fed a standard diet of Purina laboratory chow. Carbaryl was administered orally as a suspension in peanut oil at a single dosage of 60 mg/kg. Control rats received only peanut oil. Animals were killed by decapitation, and the brain was quickly removed and extracted. All determinations were made on whole brain. Serotonin was determined by the procedure of BOGDANSKI[3] and 5-HIAA by the method of GIACALONE and VALZELLI[4]. The concentration of corticosterone in the plasma was estimated by a fluorescence method[5]. The Aminco Bowman Spectrophotofluorometer was used for all fluorescent measurements.

The results of the study on the level of 5-HT and 5-HIAA in the brain are shown in the Table. The concentration of the amine and its metabolite increased significantly after 2, 4 and 6 h following carbaryl administration. After 24 h the level of both substances returned to normal.

In order to investigate the effect of carbaryl on brain 5-HT formation, endogenous stores of the amine were previously depleted by inhibition of its synthesis. Carbaryl was given 72 h after *p*-chlorophenylalanine (PCPA) administration and animals were sacrificed 4 h later. The amine level was significantly higher when compared with PCPA control value (Figure 1). Reserpine-pretreated animals showed the same trend. Plasma corticosterone level was also increased by about 125%, one hour after the oral administration of the carbamate (Figure 2).

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[1] A. HASSAN and C. CUETO JR., Z. Naturforsch. *25b*, 521 (1970).
[2] A. HASSAN, Biochem. Pharmac. (1971).
[3] D. F. BOGDANSKI, A. PLETSCHER, B. B. BRODIE and S. UDENFRIEND, J. Pharmac. exp. Ther. *117*, 82 (1956).
[4] E. GIACALONE and L. VALZELLI, J. Neurochem. *13*, 1265 (1966).
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Effect of a single carbaryl dose on brain 5-HT and 5-HIAA

Hours after Carbaryl	5-HT		5-HIAA		Ratio 5-HIAA 5-HT
	Concentration µg/g ± S.E.	% Increase	Concentration µg/g ± S.E.	% Increase	
0	0.61 ± 0.05	–	0.25 ± 0.02	–	0.41
2	0.78 ± 0.07	28	0.30 ± 0.02	20	0.38
4	0.78 ± 0.08	28	0.31 ± 0.03	24	0.40
6	0.80 ± 0.07	31	0.31 ± 0.03	24	0.39
24	0.60 ± 0.06	–	0.24 ± 0.02	–	0.40

Carbaryl dose 60 mg/kg. Results are mean of groups of 5–8 animals ± S.E.

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This probably represents the maximum elevation of the glucocorticoid. The level drops thereafter and approached pretreatment levels after 20 h. In control experiments, the administration of peanut oil alone did not cause an increase in the plasma corticosterone, after 1 h.

The effect of carbaryl on cerebral serotonin metabolism is believed to be related to a 'stress mechanism'. The effect simulates that of electric stress[6], and is presumably provoked by an enhanced synthesis and not by a decreased utilization of the amine. This assumption is supported by some complimentary evidence. 1. Serotonin level in the brain of carbaryl-treated animals, pretreated with PCPA was 72% higher than their respective controls. These results suggest that 5-HT synthesis is accelerated by carbaryl. The possibility that the effect of PCPA is diminished by carbaryl cannot be ruled out. 2. The depletion of brain 5-HT by reserpine could also be partially antagonized by carbaryl. 3. The formation of higher amounts of 5-HIAA in the brain is probably a consequence of an increased synthesis rate of serotonin, and may represent a higher functional utilization of the amine. Since organic acids are removed from the brain by an active energy-requiring process[7], an effect on transport by carbaryl could contribute to the changes in cerebral 5-HIAA levels observed.

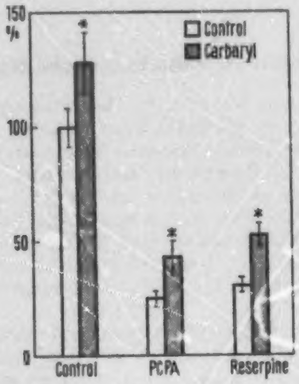

Fig. 1. Effect of carbaryl on endogenous 5-HT levels in the brain of normal and PCPA or reserpine-pretreated animals. PCPA pretreated animals received the drug i.p. (360 mg/kg) 72 h before administration of carbaryl and animals were killed 4 h later. Reserpine (5 mg/kg) was administered i.p., immediately before carbaryl was given, animals were killed 4 h later. Results are expressed as a percentage of control values ± S.E. The endogenous 5-HT levels in control animals were 0.61 ± 0.05 µg/g. $^*P < 0.01$ when compared with respective controls.

The increase in the biosynthetic rate of serotonin may involve activation of tryptophan hydroxylase and/or hydroxytryptophan decarboxylase. The possible activation of tryptophan hydroxylase receives support from the PCPA experiments. The inhibiting power of PCPA of tryptophan hydroxylase[8] could be – at least partially – overcome by carbaryl. It is also worth mentioning that stress induced by acute cold exposure significantly increases cerebral tryptophan-5-hydroxylase activity[9].

The changes in the brain amine level unlikely involve monoamine oxidase inhibition, because of the concomitant increase in cerebral 5-HIAA. In fact the ratio 5-HIAA/5-HT remained essentially unaltered (no significant differences) (Table). The administration of carbaryl into rats was reported to increase the degradation of catecholamines, presumably through activation of monoamine oxidase[3]. This suggests in turn that the increased formation of cerebral 5-HIAA in carbaryl-treated rats is probably the result of MAO activation.

The administration of carbaryl also strongly stimulates corticosterone secretion by the adrenals. Normally, corticosterone secretion would be considered an index of the release of ACTH. However, it is not known whether the changes of serotonin metabolism are related to the activation of the pituitary-adrenal system. Under conditions of restraint stress, brain 5-HT level did not correlate with the adrenocortical activation[10].

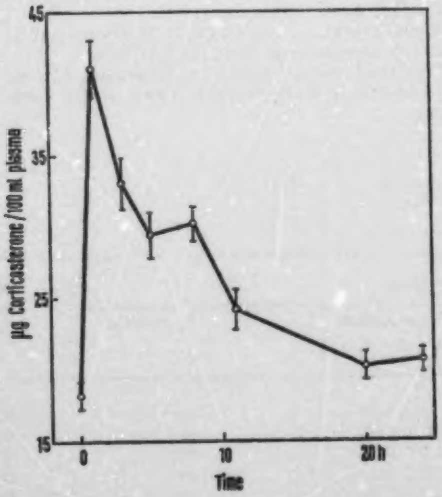

Fig. 2. Effect of a single carbaryl dose (60 mg/kg) on plasma corticosterone. Points represent means of at least 5 animals and bars indicate ± S.E.

Zusammenfassung. Nachweis, dass Carbaryl die Konzentration von Serotonin und 5-Hydroxyindolessigsäure im Rattenhirn steigert. Der Zusammenhang mit einem Stress wird als möglich angenommen.

A. Hassan[11] and J. A. Santolucito

Perrine Primate Research Branch, Division of Pesticides, Food and Drug Administration, HEW, Perrine (Florida, USA), 29 September 1970.

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National Communicable
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Atlanta, Ga. 30333

9. *Chlorinated Insecticides in Human Serum.* WILLIAM E. DALE, AUGUST CURLEY, CIPRIANO CUETO, and WAYLAND J. HAYES, JR., Toxicology Section, Communicable Disease Center, Public Health Service, U.S. Department of Health, Education, and Welfare, Atlanta, Georgia.

The concentration and distribution of chlorinated insecticides and related materials in the blood of 10 women who had not had any occupational exposure to insecticides were determined by electron capture gas chromatography. The hexane-extractable chlorinated insecticides and related materials found in serum were p,p'-DDT (0.0077 ppm), o,p'-DDT (0.0016 ppm), p,p'-DDE (0.0152 ppm), β-BHC (0.0042 ppm), dieldrin (0.0013 ppm), and heptachlor epoxide (0.0008 ppm). The mean concentrations of these materials found in serum were greater than those found in the plasma, red blood cells, or whole blood. A comparison of the levels of insecticides in the serum of women with those of men (Dale *et al.*, *Life Sci.* in press) showed that men had statistically greater concentrations of p,p'-DDT (0.0105 ppm) but lower concentrations of o,p'-DDT (0.0009 ppm) than women. Even though the differences were statistically significant ($P < 0.025$ and < 0.01, respectively), they may have been due to sampling.

Methods for the extraction of bound chlorinated insecticides and related materials in blood were also investigated.

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Toxicological Assessment of Hexachlorobiphenyl Isomers and 2,3,7,8-Tetrachlorodibenzofuran in Chicks

II. Effects on Drug Metabolism and Porphyrin Accumulation[1]

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HINDA BERGMAN AND JOHN A. MOORE[3]

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Toxicological Assessment of Hexachlorobiphenyl Isomers and 2,3,7,8-Tetrachlorodibenzofuran in Chicks. II. Effects on Drug Metabolism and Porphyrin Accumulation. Goldstein, Joyce A., McKinney, James D., Lucier, George W., Hickman, Patricia, Bergman, Hinda and Moore, John A. (1976). *Toxicol. Appl. Pharmacol.* 36, 81–92. Pure hexachlorobiphenyl (HCB) isomers induce a number of changes in parameters of drug metabolism in the chick including changes in cytochrome P-450, liver weight, and *p*-nitrophenol glucuronyl transferase, but not in testosterone glucuronyl transferase activity. The most active inducers of drug metabolism were 2,3,4,2′,3′,4′-HCB and 2,4,6,2′,4′,6′-HCB, while 2,4,5,2′,4′,5′-HCB produced intermediate effects and 2,3,6,2′,3′,6′-HCB was a poor inducer. All HCBs caused uroporphyrin accumulation and increased δ-aminolevulinic acid (ALA) synthetase activity, but only 3,4,5,3′,4′,5′-HCB, 2,3,4,2′,3′,4′-HCB, and 2,4,5,2′,4′,5′-HCB produced gross accumulation of hepatic porphyrins. Tissue HCB concentrations correlated well with hepatic effects. 2,3,7,8-Tetrachlorodibenzofuran (TCDF), a contaminant of commercial polychlorinated biphenyl (PCB) mixtures, had no effects on hepatic ALA synthetase activity, porphyrin accumulation, or glucuronyl transferase. TCDF did produce a slight increase in cytochrome P-450, but the increase was smaller than that produced by HCBs. Also, the cytochrome induced by TCDF appeared qualitatively different from the cytochrome induced by HCBs, differing by a shift in the peak of the CO-difference spectrum from 450 to 449 nm and by the ratio of absorbance at 455 to 430 nm of the ethyl isocyanide difference spectrum.

Polychlorinated biphenyls (PCBs) are industrial chemicals which are widely distributed in the environment. They have been found in tissues of wildlife and humans (Risebrough *et al.*, 1968; Biros *et al.*, 1970). Commercial mixtures of PCBs have been shown to increase liver weight, cytochrome P-450 and activity of a number of drug-metabolizing

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[1] A preliminary report of this work was presented at the Fall meeting of the American Society for Pharmacology and Experimental Therapeutics, Montreal, Canada, August, 1974 (*The Pharmacologist* 16, 239, 1974).

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enzymes including glucuronyl transferase, and to produce experimental hepatic porphyria (Litterst *et al.*, 1972; Vos *et al.*, 1971; Goldstein *et al.*, 1974a).

In general, the stimulatory effect of PCB mixtures on hepatic enzymes appears to be related to the chlorine content of the biphenyl, since potency increases with increasing percent of chlorination (Litterst *et al.*, 1972). However, two PCB mixtures with identical chlorine content by weight but different isomer composition (Aroclor 1242 and Aroclor 1016) are quite different in their effects on microsomal enzymes (Goldstein *et al.*, 1975). Little work has been done thus far comparing effects of isomerically pure chlorobiphenyl compounds. Johnstone *et al.* (1974) recently compared a number of chlorinated biphenyls in the rat at a single-dose level and found 4,4-di-, 2,4,2′,4′-tetra-, 2,4,5,2′,4′,5′-, 2,3,5,2′,3′,5′-, and 2,4,6,2′,4′,6′-hexa-, 2,3,4,5,2′,3′,4′,5′-octa-, and 2,3,4,5,6,2′,3′,4′,5′,6′-decachlorobiphenyls to be extremely active inducers of a number of drug-metabolizing enzymes. However, 2,2′- and 2,4′-dichlorobiphenyl were considerably less active, as was 2,5,2′,5′-tetrachlorobiphenyl, indicating that the position of chlorines on the biphenyl is important for activity. Fujita *et al.* (1971) found 2,4,5,3′,4′-penta and 3,4,5,3′,4′,5′-hexachlorobiphenyls to be more active inducers of hepatic drug-metabolizing enzymes than 4,4′-di- and 3,4,2′,4′-tetrachlorobiphenyls, suggesting that the number of chlorines per biphenyl may be important.

Complicating the situation even more, several commercial preparations of PCBs contain traces of the highly toxic chlorinated 2,3,7,8-tetrachlorodibenzofuran (TCDF) (Vos *et al.*, 1970). The chlorinated dibenzo-*p*-dioxins and dibenzofurans have been reported to increase both drug-metabolizing enzymes and δ-aminolevulinic acid (ALA) synthetase, the rate-limiting enzyme in heme and porphyrin synthesis (Lucier *et al.*, 1973; Poland and Glover, 1973), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCCD) has been found to be porphyrogenic to mammals (Goldstein *et al.*, 1973). Little work has been done on the mammalian effects of TCDF.

The present study was undertaken to compare effects of several hexachlorobiphenyl isomers on a number of parameters of hepatic function in the chick, including induction of hepatic drug-metabolizing enzymes and precipitation of hepatic porphyria. These effects were compared with those of TCDF.

METHODS

Chemicals. 2,4,5,2′,4′,5′-Hexachlorobiphenyl (HCB) (>99% pure), 2,3,6,2′,3′,6′-HCB (>99% pure), and 3,4,5,3′,4′,5′-HCB (66% pure) were synthesized at NIEHS (McKinney *et al.*, 1976). The impurity in 3,4,5,3′,4′,5′-HCB was 2,3,4,5,3′,4′,5′-heptachlorobiphenyl. A second batch of 3,4,5,3′,4′,5′-HCB (99% pure) was later synthesized by McKinney *et al.* (1976) by an alternate method. 2,4,6,2′,4′,6′-HCB (>99% pure) and 2,3,4,2′,3′,4′-HCB (>99% pure) were purchased from Analabs, Inc. TCDF (88% pure) was synthesized by Dr. A. S. Kende, University of Rochester, Rochester, New York. The contaminant was 1,2,3,7,8-pentachlorodibenzofuran.

Animals. One-day-old White Leghorn cockerels were obtained from Central Carolina Farmers, Durham, North Carolina. In the first experiment, the chicks were fed a diet formulated for chick edema bioassay from Nutritional Biochemicals, Cleveland, Ohio, containing 0 or 400 ppm of each of the hexachlorobiphenyls listed above or dosed orally with 1 or 5 μg/kg/day of TCDF in 0.1 ml of acetone:corn oil (1:7). The 3,4,5,3′,4′,5′-isomer used was from the first synthesis (66% pure). Another group of chicks received 0.1 ml of acetone:corn oil (1:7). The initial number of birds per group varied from 6 to 10. Cage temperature was maintained at 30°C for 2 weeks, then lowered to room temperature (25°C) at the beginning of week 3. After 3 weeks, surviving chicks were killed by carbon dioxide anesthesia. Liver enzymes were determined on four to five animals per group, and liver porphyrins on all animals. In a second experiment, chicks were fed 0, 100, and 300 ppm of both 66 and 99% pure 3,4,5,3′,4′,5′-HCB (10 chicks per group).

Liver. A portion of the liver was excised, homogenized in ice-cold 0.2 M HEPES buffer, pH 7.4, microsomes prepared (Goldstein *et al.*, 1974a), and the latter resuspended in 0.2 M HEPES buffer for assay of glucuronyl transferase, or in 1.15% KCl containing 0.1 M K_2HPO_4–KH_2PO_4 buffer (pH 7.4) and 25% glycerol for cytochrome P-450 analysis. The resuspended pellet was stored overnight at 0°C before determination of cytochrome P-450 and ethyl isocyanide ratios.

Enzyme assays. ALA synthetase activity was assayed in whole homogenates of a second portion of liver (Goldstein *et al.*, 1974a). *p*-Nitrophenol glucuronyl transferase (Lucier *et al.*, 1971) and testosterone glucuronyl transferase activities (Lucier, 1974) were determined in Triton X-100 treated microsomes.

Microsomal cytochromes and protein. Microsomal protein was measured by the method of Lowry *et al.* (1951) using crystalline albumin as the standard. Microsomal cytochrome P-450 (Omura and Sato, 1964) and ethyl isocyanide ratios (Sladek and Mannering, 1966) were measured using a DW-2 spectrophotometer.

Porphyrins. Total liver porphyrins were determined by extraction into 0.9 M perchloric 1-1 ethanol (1:1, v/v) and measured fluorometrically with uroporphyrin as a standard (Abritti and De Matteis, 1971–1972). Liver porphyrins of some samples were extracted from 0.5 to 1 g of liver, separated by thin-layer chromatography (Doss, 1967), and quantitated as previously described (Goldstein *et al.*, 1974a).

Tissue residue analysis. Subcutaneous adipose tissue and liver samples were pooled within each treatment group, extracted, and hexachlorobiphenyl residue concentrations determined by gas–liquid chromatography with a flame ionization detector (McKinney *et al.*, 1976).

RESULTS

Chicks fed 400 ppm of 66%, 3,4,5,3′,4′,5′-HCB or dosed orally with 5 μg/kg of TCDF died before sacrifice, preventing determination of their effects on liver enzymes. Results in Fig. 1 show that all other hexachlorobiphenyl isomers tested increased cytochrome P-450 content of chick livers (4- to 14-fold). 2,3,4,2′,3′,4′-HCB produced significantly greater and 2,3,6,2′,3′,6′-HCB significantly less effects than other isomers. TCDF (1 μg/kg) increased cytochrome P-450 significantly over corn oil-treated controls (threefold), but less than all HCB isomers other than 2,3,6,2′,3′,6′-HCB. The peak of the CO-difference spectrum was shifted from 450 nm for controls to 449 nm by TCDF, but remained at 450 nm in the livers of PCB-treated animals.

Liver weight was increased significantly by all PCBs except 2,3,6,2′,3′,6′-HCB (Fig. 1). The pattern was slightly different than for cytochrome P-450, in that 2,3,4,2′,3′,4′-HCB no longer produced the greatest effects. The largest increase was produced by

2,4,6,2′,4′,6′-HCB ,which was significantly greater than that produced by 2,4,5,2′,4′,5′-HCB or 2,3,6,2′,3′,6′-HCB. The second largest increase (2,3,4,2′,3′,4′-HCB) was significantly larger than that produced by 2,3,6,2′,3′,6′-HCB. TCDF did not significantly increase liver weight.

The sequence of effects on microsomal protein were similar to those on cytochrome P-450, 2,3,4,2′,3′,4′-HCB producing the largest increase and 2,3,6,2′,3′,6′-HCB the least (Fig. 1). Only 2,3,4,2′,3′,4′-HCB, 2,4,6,2′,4′,6′-HCB, and TCDF produced significant increases. The effects of 2,3,4,2′,3′,4′-HCB were significantly greater than those of 2,3,6,2′,3′,6′-HCB or 2,4,5,2′,4′,5′-HCB.

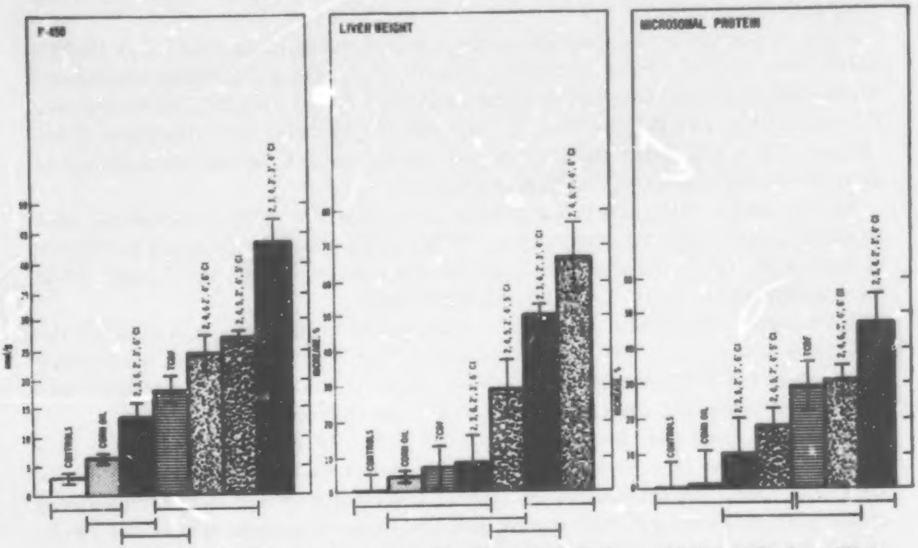

FIG. 1. Effects of purified hexachlorobiphenyl isomers and TCDF on chick livers. The hexachlorobiphenyl isomers indicated above were fed to day-old chicks at a dietary concentration of 400 ppm for 21 days. TCDF was given orally at a dose of 1 μg/kg daily for 21 days in acetone:corn oil (1:7). Controls for this group were dosed daily with acetone:corn oil (1:7). Vertical bars represent the means ± SE. Control values for liver weight were 7.3 ± 0.4 g and for liver microsomal protein were 23.9 ± 1.9 mg/g. Results were analyzed by Duncan's Multiple-Range test (Steel and Torrie, 1960), and the heavy lines below the bar graph indicate groups which do not significantly differ from each other ($p < 0.05$). Groups not underlined by the same line are significantly different.

All PCBs increased *p*-nitrophenol glucuronyl transferase activity (14- to 23-fold) (Fig. 2). Again the smallest increase was seen with 2,3,6,2′,3′,6′-HCB and the largest increase with 2,3,4,2′,3′,4′-HCB. Unlike *p*-nitrophenol glucuronyl transferase activity, testosterone glucuronidation was not affected by any of the compounds tested.

Table 1 shows the effects of hexachlorobiphenyl isomers and TCDF on the ethyl isocyanide ratios of chick liver microsomes. None of the HCB isomers had any effect on the ethyl isocyanide ratios, but TCDF did increase the ratio slightly.

As shown in Table 2, all PCBs tested produced similar increases in ALA synthetase activity (five- to six-fold), unlike the differential effects on liver weight and cytochrome

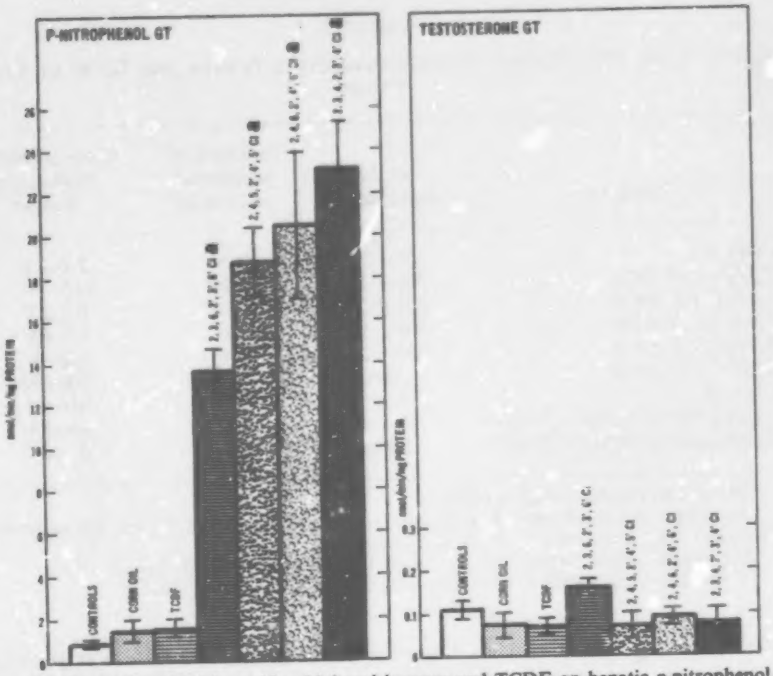

FIG. 2. Effects of purified hexachlorobiphenyl isomers and TCDF on hepatic *p*-nitrophenol and testosterone glucuronyl transferase activities. Day-old male chicks were fed 400 ppm of the above hexachlorobiphenyls or dosed orally with 1 μg/kg of TCDF in acetone:corn oil (1:7) for 21 days. Vertical bars represent the mean ± SE. [a] Significantly different from controls; $p < 0.05$ (Student's *t* test).

TABLE 1

EFFECTS OF HEXACHLOROBIPHENYL ISOMERS AND TCDF ON ETHYL ISOCYANIDE DIFFERENCE SPECTRA IN THE CHICK[a]

Treatment	455/430 ratio
Controls	0.42 ± 0.01
2,4,5,2',4,'5',-HCB	0.40 ± 0.04
2,3,6,2',3',6'-HCB	0.40 ± 0.10
2,4,6,2',4',6',-HCB	0.37 ± 0.01
2,3,4,2',3',4'-HCB	0.43 ± 0.01
Corn oil	0.51 ± 0.07
1 μg/kg of TCDF	0.74 ± 0.06[b]

[a] Treatment is described in the legend of Fig. 1. Values represent the means ± SE.

[b] Significantly different from controls. $p < 0.05$ (Duncan's Multiple-Range test).

TABLE 2

EFFECT OF 400 PPM PURIFIED HEXACHLOROBIPHENYL ISOMERS AND TCDF ON CHICK LIVERS[a]

Treatment	ALAS (nmol/g/hr)	Incidence of porphyria[b] (>10 μg/g)	Liver porphyrin levels (μg/g) (range)
Control	55 ± 2	0/10	0.4–1.4
2,3,6,2′,3′,6′-HCB	261 ± 15[c]	0/10[d]	0.5–1.4
2,4,6,2′,4′,6′-HCB	303 ± 15[c]	0/10[d]	0.7–2.9
2,4,5,2′,4′,5′-HCB	309 ± 13[c]	3/9[d]	1.5–82
2,3,4,2′,3′,4′-HCB	250 ± 28[c]	2/8[d]	1.2–81
3,4,5,3′,4′,5′-HCB	Dead	1/7[d]	2.4–5000
Corn oil	74 ± 2	0/3	0.4–1.2
1 μg/kg/day of TCDF × 21 days	63 ± 20	0/5	0.4–2.9
5 μg/kg/day of TCDF × 21 days	Dead	0/5	0.3–0.7

[a] Treatment described in legend of Fig. 1.

[b] Proportion of animals whose liver porphyrin concentrations exceed 10 μg/g. The upper 99% tolerance levels for controls at $p < 0.01$ is 2.3 μg/g.

[c] Significantly greater than controls, $p < 0.05$ (Duncan's Multiple-Range test).

[d] Uroporphyrin detectable in all samples after thin-layer chromatography.

P-450. In contrast, TCDF produced no effect. Liver porphyrins were determined in all groups, including animals which died or were killed or moribund before 21 days. Table 2 shows the incidence of porphyria, defined as a porphyrin concentration greater than 10 μg/g of liver (control = 0.8 ± 0.1 μg/g), and the range of each group. The upper tolerance limit for 99% of control samples at $p < 0.01$ is 2.3 μg/g. Many of the animals in all groups were within the tolerance limits for control samples. However, 3/9 of the samples fed 400 ppm 2,4,5,2′,4′,5′-HCB, 2/8 of the samples fed 2,3,4,2′,3′,4′-HCB, and 1/7 of the samples fed 3,4,5,3′,4′,5′-HCB (died or were killed moribund before 21 days) were grossly porphyric. Although no animals fed 2,3,6,2′,3′,6′-HCB were porphyric, neither were any animals fed 2,4,6,2′,4′,6′-HCB porphyric. Extraction, purification, and thin-layer chromatography were performed on two to three liver samples from each treatment group. Only traces of protoporphyrin and coproporphyrin were found in control livers, but traces of 8- and 7-carboxyporphyrins were seen in all PCB-treated livers analyzed, even from those animals fed 2,3,6,2′,3′,6′-HCB and 2,4,6,2′,4′,6′-HCB. Only 8- and 7-carboxyporphyrins could be detected in porphyric livers. Porphyrin also accumulated in the crop and bones. The porphyrin in the crop was 4-, 7-, and 8-carboxyporphyrins (31, 20, and 49%, respectively), while the porphyrin in the bones was entirely 7- and 8-carboxyporphyrins (27 and 73%). None of the animals fed 1 or 5 μg/kg of TCDF showed any accumulation of porphyrins, even after extraction and thin-layer chromatography.

In the second experiment, chicks fed 0, 100, or 300 ppm of 66% pure (crude) versus 99% pure (pure) 3,4,5,3′,4′,5′-HCB died before 21 days; therefore, effects on liver enzymes were not determined. Liver porphyrins were elevated by both pure and crude 3,4,5,3′,4′,5′-HCB at both dietary levels (Table 3).

TABLE 3

EFFECT OF 3,4,5,3',4',5'-HEXACHLOROBIPHENYL ON HEPATIC PORPHYRINS[a]

Treatment	Incidence of porphyria[b]	Liver porphyrins[c] (μg/g)
Control	0/10	0.66 ± 0.07
100 ppm 3,4,5,3',4',5'-HCB(66% pure)	5/8	16.7 ± 11.2[d]
300 ppm 3,4,5,3',4',5'-HCB(66% pure)	7/7	200 ± 53[d]
100 ppm 3,4,5,3',4',5'-HCB(99% pure)	8/8	68 ± 42[d]
300 ppm 3,4,5,3',4',5'-HCB(99% pure)	7/7	267 ± 127[d]

[a] Two synthetic batches of 3,4,5,3',4',5'-HCB (66% and 99% pure) were fed to chicks at the dietary concentrations indicated for 21 days. All chicks fed 100 or 300 ppm of either batch of the HCB died before 21 days, and liver samples were taken at autopsy.

[b] Proportion of animals whose liver porphyrins exceed the upper 99% tolerance limit for controls at $p < 0.01$ (1.75 μg/g).

[c] Values represent means ± SE.

[d] Significantly greater than controls, $p < 0.05$ (Student's *t* test).

Toxicity expressed as death (groups fed 400 ppm 3,4,5,3',4',5'-HCB or dosed orally with 5 μg/kg/day of TCDF) or body weight loss (Fig. 3) appears unrelated to effects on the liver. 2,4,5,2',4',5'-HCB and 2,3,4,2',3',4'-HCB caused significant weight loss, while 2,4,6,2',4',6'-HCB produced no weight loss.

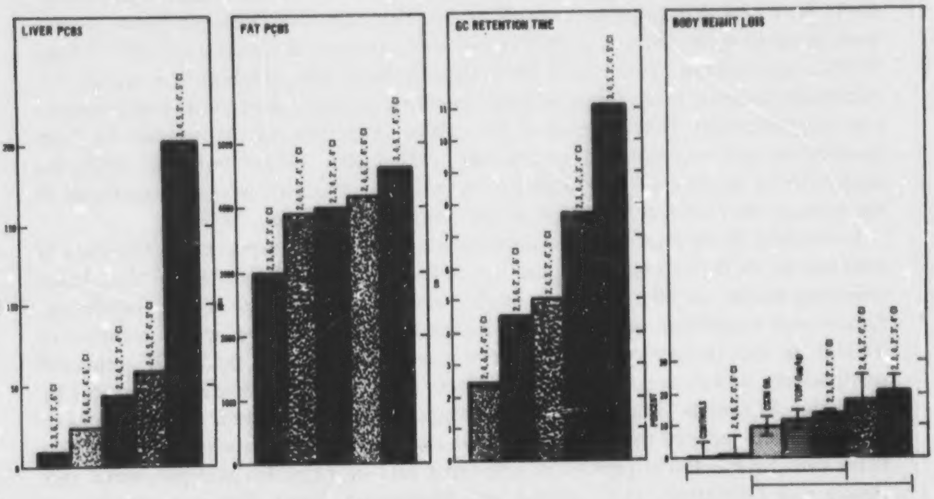

FIG. 3. Liver and fat concentration of hexachlorobiphenyls and body weight loss of chicks fed purified hexachlorobiphenyl isomers. Chicks were treated as described in Fig. 1. Values for liver and fat concentrations of PCBs are the results of duplicate analyses of pooled liver samples. Body weight loss is reported as percentage of control of body weight (203 ± 10 g). Values represent means ± SE, and heavy lines beneath bars indicate groups which do not differ significantly, $p < 0.05$ (Duncan's Multiple-Range test).

The PCB concentration of fat paralleled effects on liver weight (Fig. 3). 3,4,5,3',4',5'-HCB which was lethal at 400 ppm in the diet, produced the highest liver and fat PCB content. The 2,3,6,2',3',6'-isomer accumulated the least and produced the least liver effects. GC retention times (Fig. 3) correlate reasonably well with HCB content of fat, except for the relatively low retention time and high fat levels and biological activity of 2,4,6,2',4',6'-HCB.

DISCUSSION

Our results show that pure hexachlorobiphenyls alter a number of parameters of hepatic function in the chick, including cytochrome P-450, microsomal protein, liver weight, and glucuronyl transferase activity. There were distinct differences in the induction produced by the hexachlorobiphenyl isomers tested: 2,3,4,2',3',4'-HCB and 2,4,6,2',4',6'-HCB were the most active inducers, 2,4,5,2',4',5'-HCB produced intermediate effects, and 2,3,6,2',3',6'-HCB was a relatively poor inducer of the above biochemical parameters. These results show that even within the highly chlorinated hexachlorobiphenyls, position of the chlorines is important for activity. The relative inactivity of the 2,3,6,2',3',6'-hexa- isomer indicated that occupation of the *p*-position may be important. The work of Johnstone *et al.* (1974) also indicated that occupation of the *para*-positions may be a very important factor governing ability to induce liver enzymes, especially with PCB isomers with low chlorine content. Differences among the more potent hexa- isomers were not obvious, probably because the dose utilized produced a maximum increase. An additional lower dose of the hexachlorobiphenyls might have revealed differences in this group. The differences between hexa- isomers were possibly more obvious in the present study because the increase in cytochrome P-450 is much greater in the chick (14×) than in the rat (4×) (Goldstein *et al.*, 1974a). Although glucuronyl transferase activity in microsomes of control chicks was extremely low, approximately 1/30 of those of rat, rabbit, or guinea pig microsomes for both testosterone and *p*-nitrophenol (Lucier *et al.*, 1971; Lucier, 1974; Hook *et al.*, 1975), the magnitude of the increase produced by the hexachlorobiphenyl isomers was similar to the increase produced by PCB mixtures in rats (Goldstein *et al.*, 1975).

In contrast to the purified hexachlorobiphenyls, TCDF, a suspected contaminant of commercial PCB mixtures, produced less induction of cytochrome P-450 than hexachlorobiphenyls, no effect on liver weight, and no effect on glucuronyl transferase. Glucuronyl transferase activity was stimulated markedly by the related compound, TCDD, in rats (Lucier *et al.*, 1973). The cytochrome induced by TCDF appeared qualitatively, as well as quantitatively, different from the cytochrome P-450 induced by hexachlorobiphenyls. Similarly, administration of certain polycyclic hydrocarbons or TCDD to rats induced the formation of cytochrome P-448, a cytochrome that differs from cytochrome P-450 present in untreated rats or phenobarbital-pretreated rats (Greig and DeMatteis, 1973; Sladek and Mannering, 1966). The new cytochrome differed from cytochrome P-450 by a 2-nm shift in the peak of the CO-difference spectrum, increase in the ratio of the 455 to 430 nm peaks of the ethyl isocyanide difference spectrum, and the substrate specificity. Administration of PCBs, on the other hand, appeared to increase formation of both cytochromes P-450 and P-448 in rats: the peak of the CO-difference spectrum was shifted to 448 nm, but the ratio of the

455 nm to the 430 nm peaks of the ethyl isocyanide difference spectrum was changed to a value intermediate between those found after treatment with polycyclic hydrocarbons and phenobarbital, and there was increased metabolism of both substrates metabolized primarily by cytochrome P-450 and cytochrome P-448 (Alvares *et al.*, 1975). In the present study, only TCDF shifted the peak of the CO-difference spectrum and altered the ratio of the 455/430 nm ethyl isocyanide peaks. The apparent formation of cytochrome P-450 rather than cytochrome P-448 after administration of PCB isomers was unexpected, and may reflect either a species difference or differences between isomers. Alternately, induction of cytochrome P-448 by PCB mixtures may be a result of contamination by TCDF.

We previously reported that PCB mixtures produced hepatic porphyria, accumulation of uroporphyrins, and increased activity of ALA synthetase in the rat (Goldstein *et al.*, 1974a). Only three of the hexachlorobiphenyls tested in the present study produced an incidence of porphyria: 2,4,5,2′,4′,5′-HCB, 2,3,4,2′,3′,4′-HCB, and 3,4,5,3′,4′,5′-HCB. Interestingly, these isomers were also the most toxic, as measured by death or weight loss. It is notable that all three of these isomers had both *m*- and *p*-positions occupied by chlorines, while 2,3,6,2′,3′,6′-HCB and 2,4,6,2′,4′,6′-HCB with *p*- or *m*-positions, respectively, unoccupied were inactive. Alternatively, the nonplanarity provided by the 4 *ortho* chlorines may have decreased the porphyrogenic activity of these two compounds. However, all hexachlorobiphenyl isomers tested increased ALA synthetase and produced a slight accumulation of uroporphyrins. The time course of this disease has not been studied in chickens, but there is a lag of several weeks before porphyria and increased activity of ALA synthetase can be demonstrated in rats fed PCBs (Goldstein *et al.*, 1974a). The induction of ALA synthetase and slight increase in uroporphyrin accumulation in all chicks suggests that all hexachlorobiphenyls tested were porphyrogenic; however, there is probably a lag period in the onset of the disease, and some hexachlorobiphenyls appear more porphyrogenic than others. Reports that certain tetrachlorobiphenyl isomers did not increase ALA synthetase when administered acutely to rats (Chen *et al.*, 1973) are of doubtful significance, since similar doses of PCB mixtures did not increase ALA synthetase activity when given acutely to rats, but increased activity when fed chronically (Goldstein *et al.*, 1974a).

The presence of small amounts of TCDF in some commercial PCB mixtures (Vos *et al.*, 1970) has led many authors to speculate that the porphyrogenic effects of PCBs could be produced by contamination with TCDF. The related compound TCDD was porphyrogenic to mice at doses of 25 μg/kg/week × 4 weeks (Goldstein *et al.*, 1973), and both TCDD and TCDF have been shown to induce ALA synthetase and aryl carbon hydroxylase in the chick embryo at approx 5×10^{-11} mol/egg (Poland and Glover, 1973). The present study shows that TCDF did not produce porphyria in chicks, even at lethal doses, or increase ALA synthetase. We have also shown that TCDF does not produce accumulation of porphyrins in livers of mice at doses as high as 4 mg/kg (unpublished observations; Goldstein *et al.*, 1974b). These results suggest strongly that TCDF plays no significant role in the production of porphyria by PCB mixtures. Similarly, the relatively lesser effects of TCDF than hexachlorobiphenyls on liver enzymes make it extremely unlikely that any significant portion of the effects of PCB mixtures could be due to contamination with trace amounts of TCDF. Possibly, differences in absorption, distribution, or metabolism of TCDF versus TCDD may explain the relative inactivity of TCDF as an inducer of porphyria and drug-metabolizing enzymes in mammals and adult chickens, whereas both compounds are very potent in the chick embryo.

Tissue PCB concentrations can partially explain the differences in hexachlorobiphenyl isomers as hepatic inducers. The 3,4,5,3′,4′,5′-isomer accumulated the most in fat and liver, caused porphyria and was lethal. The 2,3,6,2′,3′,6′-isomer accumulated the least in both fat and liver, and produced the smallest increase in all parameters. However, some differences in hepatic effects (2,3,4,2′,3′,4′, > 2,4,5,2′,4′,5′) cannot be explained by liver concentrations. Concentrations of HCBs in fat correlated well with liver effects. This may suggest that lipophilicity is an important factor in hepatic potency. GC retention indices also correlated reasonably well with liver effects with the exception of 2,4,6,2′,4′,6′-HCB. This isomer and 2,3,6,2′,3′,6′-HCB both have four *ortho* chlorines which would tend to hinder rotation and make the compound nonplanar. The 2,4,6,2′,4′,6′-isomer is highly symmetrical, however, adding to its polarity. GC retention times may reflect lipophilicity with the exception of biphenyls which have four *ortho* positions occupied by chlorine. Alternatively, 2,3,6,2′,3′,6′-HCB could exist as a racemic mixture because of steric hindrance provided by the *ortho* chlorines. It is possible that one form is relatively inactive, resulting in lower activity for the mixture. Significantly, Bush *et al.* (1974) and Baker *et al.* (1974) found that tissue persistence of individual isomers in chickens fed Aroclor 1254 correlated with *para*-substitution and mixed-function oxidase activity especially for the penta- and hexa- isomer.

Tissue PCB concentrations may be an important factor in biological effectiveness. It is well known that some isomers, particularly the lower chlorinated homologs, are preferentially metabolized and excreted over highly chlorinated homologs (Weigel and Smith, 1974). On the other hand, studies with Aroclor mixtures with comparable chlorine content but differing isomer composition (i.e., Aroclors 1242 and 1016) demonstrated marked differences in biological potency despite comparable tissue concentrations (Goldstein *et al.*, 1975). The question remains as to the extent to which differences in absorption, metabolism, and distribution determine biological activity of polychlorinated biphenyl isomers. Future studies should direct further attention to the importance of the position and number of chlorines on the biphenyl nucleus in determining biological activity, and the relationship of these parameters to metabolism and tissue distribution.

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Chlorinated Insecticides in the Body Fat of People in India*

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The storage of insecticides in the body-fat of persons has previously been studied in several countries (e.g., England, France, USA) and food has been shown to be the main source from which DDT and perhaps other insecticides are absorbed. In view of the differences in dietary habits in India, a study was made to determine whether there are differences in the storage of chlorinated hydrocarbon insecticides in persons in India from that in persons in other countries.

It was found that civilians in the Delhi area with no occupational exposure to insecticides had a significantly greater deposit in their fat of DDT-related compounds and HCH than do comparable persons in the USA, but less dieldrin and heptachlor epoxide. Storage in military personnel in other Indian cities from which samples were obtained was similar to that in the USA for most DDT-related compounds and HCH but, as in Delhi, lower for dieldrin and heptachlor epoxide.

It is well known that both DDT (1,1,1-trichloro-2,2-*bis*-(*p*-chlorophenyl) ethane) and its metabolite DDE (1,1-dichloro-2,2-*bis*-(*p*-chlorophenyl) ethylene) are present in the general population of several countries (Mattson et al., 1953; Hayes et al., 1958, 1963; Hunter et al., 1963; Dale & Quinby, 1963; Hoffman et al., 1964a, 1964b). It has also been shown that dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo-exo-5,8-dimethanonaphthalene) is present in the fat of people in the USA (Dale & Quinby, 1963; Hoffman et al., 1964a, 1964b) and southern England (Hunter et al., 1963) and that HCH (1,2,3,4,5,6-hexachlorocyclohexane) is present in the body fat of people in the USA (Dale & Quinby, 1963; Hoffman et al., 1964a, 1964b) and France (Hayes et al., 1963). It has recently been shown by the use of gas chromatography that DDD (1,1-dichloro-2,2-*bis*(*p*-chlorophenyl) ethane) and the β-isomer of HCH are stored in the body fat of the general population of New Orleans (Hayes et al., 1965). Heptachlor epoxide (1,4,5,6,7,8,8-heptachloro-2,3-epoxy-3a,4,7,7a-tetrahydro-4,7-methanoindan) has also been found in the body fat of some people in the USA (Hayes et al., 1965; Zavon et al., 1965) who had no known occupational exposure.

In India, in contrast to countries where the storage of insecticides has been studied previously, there has been nation-wide use of DDT for the control or eradication of malaria and some use in other fields of public health. DDT has not been used for preservation of grains under the control of the Government, but such preservation may be practised privately; market samples have shown residues of DDT as high as 16.3 ppm in wheat and 169 ppm in potatoes (Sharangapani & Pingale, 1954; S.K. Majumder—personal communication, 1964). HCH is widely used in plant protection and animal husbandry in India. Other chlorinated insecticides, such as dieldrin and heptachlor epoxide, find use mainly in plant protection. The dietary habits also differ in India, there being a large per capita consumption of cereals and comparatively less consumption of food derived from animal sources than in the USA and Europe. The cereals also are used in the form of whole-meal flours from which bran is generally not removed.

In countries in which the storage of insecticides has been studied previously, the diet has been shown to be the main source for the absorption of DDT (and probably other insecticides also) in persons without occupational exposure. Because of the

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dietary [illegible] and other possible differences, it seemed desirable to learn whether there are any quantitative or qualitative differences in the storage of chlorinated hydrocarbon insecticides in people in India as compared with people in other countries.

METHODS

In order that the results of this survey might be compared with earlier surveys conducted in other countries, all the samples were analysed by the Schechter-Haller colorimetric method (Schechter et al., 1945) as modified by Mattson et al. (1953). The fat samples that were large enough were also analysed by microcoulometric gas chromatography (Coulson et al., 1960) to detect other chlorinated hydrocarbon pesticides.

The 104 samples of human fat reported on here were collected in the Dehli–New-Dehli, Amla, Jabalpur, Kanpur, Poona, and Bombay areas of India. The samples were taken from people of widely different ages (3-75 years) and occupations, all without any known occupational exposure to pesticides. In connexion with the 48 autopsy and 56 surgical samples collected, in no instance was death or illness related to pesticides. The samples were preserved in 4% formaldehyde (10% formol solution) during shipment. It has been shown (Hayes et al., 1958) that this method of preservation is satisfactory for such samples.

The samples were grouped according to place of origin and method of analysis. Group I included 69 samples from the vicinity of Delhi, all of which were analysed by the colorimetric method (Mattson et al., 1953). Twenty-four of the same samples were also analysed by microcoulometric gas chromatography (Coulson et al., 1960) and were designated as Group Ia. Group II included 19 fat samples collected in the Delhi area but from people whose addresses were not known. A total of 16 fat samples from military personnel from Amla, Jabalpur, Kanpur, Poona, and Bombay were designated as Group III and analysed by the colorimetric method; 11 of these were also analysed by microcoulometric gas chromatography and were designated as Group IIIa.

All the samples were ground and extracted with *n*-hexane in the presence of sodium sulfate. The extracts were then filtered, and those samples that were to be analysed by both the colorimetric method and microcoulometric gas chromatography were made to volume and aliquots taken for the colorimetric analysis. The remainder of the extract was cleaned up and prepared for gas chromatography (Dale & Quinby, 1963). In addition to the cleaning, this procedure gives a separation of DDE and dieldrin which could not be efficiently separated by the gas chromatographic column owing to the high concentration of DDE in the fat as compared with that of dieldrin.

The gas chromatographic analyses were carried out with a MicroTek 2500-R instrument equipped with a Dohrmann Microcoulometric Detector. The column consisted of an aluminium tube 4 feet by 1/8 inch external diameter (122 cm by 3.2 mm) packed with 1% Apiezion L and 0.2% Epikote 1001 on 80-100 mesh celite (Robinson, 1963). The temperature of the column was maintained at 130°C for the first 21 minutes after each sample was injected in order to separate the isomers of HCH. The oven temperature was then increased at a constant rate of 8.75°C per minute for four minutes. The column temperature was maintained at the attained temperature of 165°C for an additional 23 minutes for the elution of other pesticides. The carrier gas, nitrogen, was maintained at a flow rate of 26 ml/min, and the effluent gases were oxidatively decomposed at 900°C and titrated coulometrically.

RESULTS

Colorimetric analysis

Quantitative results obtained by the colorimetric method of analysis for DDT-derived materials in the fat of the general population of India are summarized in Table 1.

The mean for the total DDT-derived material in the 69 samples from Delhi was 32.1 ppm when the values for two children (a boy and a girl aged 3 and 7 years), who had DDT-derived material in their fat at concentrations of 291 ppm and 180 ppm, respectively, are considered with the values for the other 67 samples in Group I. The next highest concentration in the samples was 82 ppm. Since there was no way to exclude the possibility that exposure of the children was due to sources other than those associated with the other samples, they were not considered representative of the group and therefore were not included in the values reported in Table 1. There was no significant difference in DDE ($P > 0.70$) and DDT ($P > 0.20$) storage between Groups I and II. Although the addresses of individuals in Group II were not known, they were most likely from the Delhi area since the samples were picked up there

TABLE 1

DDT-DERIVED MATERIAL IN THE BODY FAT OF PEOPLE IN INDIA ESTIMATED BY SCHECHTER-HALLER COLORIMETRIC ANALYSIS

Group [a]	Statistical value	DDT (ppm)	DDE (ppm)	Total as DDT	DDE as DDT (%)
I	Range Mean ± SE N	2–57 16±1 67	<1–40 9±1 67	2–82 26±2 67	39
	Group I *versus* Group III Group I *versus* Group II	P<0.01 P>0.20	P<0.05 P>0.70	P<0.01 P>0.25	
Ia	Range Mean ± SE N	5–57 18±2 24	2–23 11±1 24	13–69 30±3 24	41
II	Range Mean ± SE N	6–91 20±4 19	<2–28 10±2 19	8–118 31±6 19	34
III	Range Mean ± SE N	1–24 8±2 16	<1–18 4±1 16	1–44 13±3 16	37
	Group III *versus* Group II	P<0.025	P<0.025	P<0.01	
IIIa	Range Mean ± SE N	1–24 7±2 11	<1–16 4±2 11	1–44 12±4 11	37

[a] For explanation of the groups, see the third paragraph under " Methods ".

and the values are statistically similar. Samples of fat in Groups I and II contained considerably more DDT-derived material than has been reported for the general population of other countries (Table 2). The results in Table 1 show that samples from Group I contained significantly more DDE ($P < 0.05$) and DDT ($P < 0.01$) than those from Group III. It was also shown that there was significantly more DDE ($P < 0.025$) and DDT ($P < 0.025$) in Group II than in Group III.

DDT was present in greater amounts than DDE as an average for each group studied and in 60 of 67 individual fat samples of Group I, in 14 of 19 samples in Group II, and in 10 of 16 samples from Group III. DDE calculated as DDT averaged 39%, 34%, and 37% of the total DDT-derived materials in Groups I, II, and III, respectively. As shown in Table 2, these percentages are considerably lower than those reported for other countries, and the difference appears to be significant.

Since there was no significant difference in the storage of DDE and DDT in the fat of individuals in Groups I and II by the colorimetric method, these 86 individuals were considered as one group for the purpose of comparison of sex, age, autopsy *versus* biopsy, and urban *versus* rural. An analysis of variance for all ages showed that there was no significant difference ($P > 0.25$) in the storage of DDT-derived materials based on age. A comparison of 41 males and 46 females showed that there was no significant difference based on sex in the storage of DDE ($P > 0.05$) or DDT ($P > 0.10$) in the fat. A comparison was made between 47 autopsy and 39 biopsy samples and there was no significant difference between the two groups of samples in the storage of DDE ($P > 0.10$) or DDT ($P > 0.50$) in the fat. There was also no significant difference in the storage of DDE ($P > 0.40$) or DDT ($P > 0.50$) based on a comparison of 25 urban with 16 rural fat samples from the Delhi area.

TABLE 2

CONCENTRATION OF DDT AND ITS METABOLITE DDE IN THE FAT OF THE GENERAL POPULATION OR OF CERTAIN DIETARY GROUPS OF VARIOUS COUNTRIES

Country	Year	No. of samples	DDT (ppm)	DDE as DDT (ppm)	Total as DDT (ppm)	DDE as DDT (%)	Reference
USA	1955	49	7.4	12.5	19.9	62.8	Hayes et al. (1956)
USA	1954-56	61	4.9	6.8	11.7	58.1	Hayes et al. (1958)
USA	1961-62	130	4.0	8.6	12.6	68.3	Quinby et al. (1965)
USA	1963	28	2.4	4.2	6.7	62.0	Dale & Quinby (1963)
USA	1963	282	2.9	8.2	11.1	73.9	Hoffman et al. (1964a, 1964b)
USA [a]	1955-56	16	2.3	3.6	5.9	61.0	Hayes et al. (1958)
USA [b]	1960	20	0.8	2.2	3.0	73.3	Durham et al. (1961)
Canada	1959-60	62	1.6	3.3	4.9	67.0	Read & McKinley (1961)
Germany	1958-59	60	1.0	1.3	2.3	56.5	Maier-Bode (1960)
France	1961	10	1.7	3.5	5.2	67.3	Hayes et al. (1963)
India, Group I	1964	67	15.9	10.1	26.0	39.0	This article
India, Group II	1964	19	20.3	10.7	31.0	34.0	This article
India, Group III	1964	16	8.1	4.7	12.8	37.0	This article

[a] Persons eating no meat.
[b] Eskimos (Alaska).

Microcoulometric gas chromatography

The system described above readily determined quantitatively 0.1 μg of a number of compounds or isomers with characteristic retention times as follows: α-HCH, 7.5; γ-HCH, 10.7; β-HCH, 16.6; δ-HCH, 19.0; heptachlor epoxide, 26.5; *o,p′*-DDE, 28.0; dieldrin, 29.4; *p,p′*-DDE, 31.6; *o,p′*-DDT, 35.5; *p,p′*-DDD, 38.6; *p,p′*-DDT, 43.4; and endrin with two peaks, 62.4 and 76.4 minutes. The α and β isomers of HCH, dieldrin, *p,p′*-DDE, *o,p′*-DDT, *p,p′*-DDD, and *p,p′*-DDT were found in some or all of the 35 fat samples subjected to gas chromatography. The α-isomer of HCH was present in 30 of the 35 samples, and the β-isomer in all of them. Dieldrin was found in 24 of the 35. The *p,p′*-isomers of DDT, DDD, and DDE appeared in all samples analysed; the *o,p′*-DDT isomer was present in 21 of the 35 samples. Quantitative results obtained by gas chromatography for DDT-derived materials and other chlorinated hydrocarbons are summarized in Table 3.

Statistical evaluation of results reported in Tables 1 and 3 showed that there was no significant difference ($P > 0.80$) in the storage of total DDT-derived material in the fat based on the two methods of analysis.

Group IIIa contained significantly less *p,p′*-DDE ($P < 0.05$); *p,p′*-DDT ($P < 0.025$); and total DDT ($P < 0.025$) than Group Ia. Individuals in Group IIIa also contained significantly less α-HCH ($P < 0.001$) and β-HCH ($P < 0.001$) in their fat than those in Group Ia. There was, however, no significant difference in the storage of *p-p′*-DDD ($P > 0.02$), *o,p′*-DDT ($P > 0.10$) and dieldrin ($P > 0.20$) between the two groups.

By means of gas chromatography DDE calculated as DDT averaged 42% and 52% of the total DDT-related materials in Groups Ia and IIIa, respectively. DDD responds to the colorimetric method to give the same blue colour as that of DDT. Therefore, DDD was undoubtedly a factor contributing to the low average percentage of DDE of the total DDT-related materials found in the fat by colorimetric analysis. Furthermore, DDD is manufactured as an insecticide in its own right and, therefore, is not necessarily derived from DDT through metabolism.

TABLE 3. CONCENTRATION OF CHLORINATED HYDROCARBON PESTICIDES IN THE BODY FAT OF PEOPLE IN INDIA COMPARED WITH THAT OF PEOPLE IN NEW ORLEANS, LOUISIANA, AS MEASURED BY MICROCOULOMETRIC GAS CHOMATOGRAPHY

Location and group [a]	Statistical value	*p, p'*-DDE (ppm)	*o, p'*-DDT (ppm)	*p, p'*-DDD (ppm)	*p, p'*-DDT (ppm)	Total as DDT (ppm)	DDE as DDT (%)	α-HCH (ppm)	β-HCH (ppm)	Total as HCH (ppm)	Dieldrin (ppm)
India, Group Ia	Range Mean±SE N	2–23 11.8±1.2 24	<0.06–4.1 0.79±0.25 24	0.5–6.5 2.7±0.4 24	1.3–51.3 13.5±2.4 24	6.80–71.5 30.2±3.8 24	43	<0.03–0.77 0.28±0.37 24	0.17–3.16 1.30±0.17 24	0.30–4.9 1.7±0.23 24	<0.02–0.38 0.08±0.01 24
India, Group IIIa	Range Mean±SE N	0.41–23.9 6.4±2.1 11	0.2–2.33 0.8±0.2 11	0.2–6.90 1.7±0.6 11	0.2–11.3 3.8±1.2 11	0.8–47.90 13.7±4.2 11	52	0.04–0.14 0.06±0.01 11	0.13–3.06 0.80±0.25 11	0.13–3.18 0.86±0.20 11	0.01–0.19 0.06±0.01 11
	Group IIIa *versus* Group Ia	P<0.05	P>0.10	P>0.20	P<0.025	P<0.025		P<0.001	P<0.001	P<0.01	P>0.20
New Orleans [b]	Range Mean±SE N	0.56–21.17 6.65±0.8 25	<0.01–0.67 0.06±0.03 25	0.09–0.99 0.45±0.05 25	0.15–5.99 1.73±0.26 25	1.1–27.09 10.3 ±1.1 25	77	<0.03	0.03–2.43 0.80±0.10 25	0.03–2.43 0.80±0.10 25	0.03–1.15 0.29±0.05 25
	Group IIIa *versus* New Orleans	P>0.80	P<0.001	P<0.005	P>0.10	P>0.05			P>0.40	P>0.80	P<0.01

[a] For explanation of the groups, see the third paragraph under " Methods ".

[b] The fat of the people of New Orleans also contained heptachlor epoxide (0.24±0.05 ppm) and *o, p'*-DDE (0.29±0.03 ppm). The *o, p'*-DDE is included in the total as DDT in the above tabulation.

On this basis, DDE calculated as DDT averaged 45% and 60% of the total DDT-derived materials in the fat of Groups Ia and IIIa, respectively. When calculated in a similar manner, the DDE concentration with respect to the total DDT-derived materials in the samples from the New Orleans survey mentioned below was 80%.

Regardless of any consideration of DDD, there was more DDT than *p,p'*-DDE in 13 of 24 of the samples in Group Ia. However, the absolute amount of *p,p'*-DDE stored by this group is greater than that in New Orleans (see Table 3) and, in fact, in the USA as a whole (Dale & Quinby, 1963). Since the total amount of DDT-derived material present in the fat of these individuals was greater than that of the general population of other countries (Table 2), more extensive exposure is indicated. Really high storage of DDT has been associated with low percentages of DDE. For example, formulators of insecticide preparations whose storage of DDT-related material varied from 264 ppm to 1131 ppm had only 54%-40% of it in the form of DDE (Mattson et al., 1953; Hayes et al., 1956). Furthermore, since most Indians eat little meat, they most likely would be getting a relatively low proportion of DDE in their diet, because the metabolism of DDT by animals tends to increase the proportion of DDE above that originally absorbed. Thus, both a high absolute intake of DDT and a low proportion of DDE in the diet may contribute to the fact that in India the average percentage of DDE among the total DDT-derived materials is low compared with the corresponding percentages in other countries. The high concentration of DDD in Indian samples is responsible for the fact that the proportion of DDE is lower when calculated on the basis of results obtained by the Schechter-Haller method, because by that method DDD is not measured separately but recorded as DDT.

A similar procedure of microcoulometric gas chromatography was used in a recent survey of fat from individuals of New Orleans, Louisiana. The results of that survey are included in Table 3 for comparison. Since the concentration of pesticides in the fat of the Indians in Group IIIa was equal to or lower than that of Group Ia, the statistical comparison was made between Group IIIa and the New Orleans group. Of the materials found to be present in the fat of the Indians, those in Group IIIa stored significantly less dieldrin (P <0.01) and significantly more *p,p'*-DDD (P <0.005) and *o,p'*-DDT (P <0.001) than people from New Orleans. There was no significant difference between the storage of the β-isomer of HCH in the fat of Indians in Group IIIa and that in the fat of people in New Orleans. There was, however, a difference in the storage of heptachlor epoxide, which was not found in any Indian sample, and the α-isomer of HCH, which was not found in any sample from New Orleans. Since only trace amounts of the α-isomer were found in Indian fat of Group IIIa, there was no significant difference ($P<0.20$) in the storage of total HCH between this group and that from New Orleans. The total HCH stored in the fat of Indians in Group Ia (Delhi) is not significantly different ($P>0.20$) from that reported for France (Hayes et al., 1963), but is significantly greater ($P<0.001$) than that reported for the USA (Dale & Quinby, 1963). These data show that the general population of Delhi has significantly greater amounts of DDD, DDT-derived materials, and HCH but less dieldrin and less heptachlor epoxide in their fat than the general population of New Orleans. A comparison between 17 males and seven females was made for the storage of dieldrin and total HCH in the fat of Indians. There was no significant difference by sex in the storage of dieldrin ($P>0.30$) or HCH ($P>0.50$).

DISCUSSION

The frequency of high residues of DDT in samples of food taken on the open market in India is not yet known. However, some high residues have been encountered and this finding is entirely consistent with the finding that people of the Delhi area store more HCH and more DDT-related material than do people in the USA. The major remaining problem is to identify the difference in practice which accounts for the observed difference in storage in the Delhi area as compared with other cities of India from which samples were obtained. It seems possible that the difference concerns the extent of use of insecticides for the preservation of stored food in the Delhi area as compared with other cities that have been studied, or the difference may involve civilian as opposed to military practices since the samples from other cities came from soldiers or members of their families.

The finer points of difference in storage, including the presence of α-HCH and the greater concentration of *o,p′*-DDT and *p,p′*-DDD in all samples from India as compared with samples from New Orleans cannot be explained at this time.

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RÉSUMÉ

Dans différents pays, des études ont déjà été consacrées au problème de l'accumulation des insecticides dans le tissu adipeux de l'homme. Elles montrent que ces dépôts, ceux de DDT notamment, ont une origine principalement alimentaire. Les auteurs ont effectué une enquête similaire en Inde afin de rechercher une influence éventuelle des habitudes alimentaires des populations de ce pays sur l'accumulation dans l'organisme des hydrocarbures chlorés.

Au cours de nécropsies ou d'interventions chirurgicales, 104 échantillons de tissu adipeux humain ont été prélevés chez des personnes de 3 à 75 ans, non exposées professionnellement à un risque inhabituel d'absorption d'insecticides. L'examen de ces préparations a été effectué par la méthode colorimétrique ou par chromatographie gazeuse. Il a permis de déceler la présence des α- et β-HCH, de la dieldrine, de l'isomère *p,p′* du DDD, des isomères *o,p′* et *p,p′* du DDT, de même que celle de l'isomère *p,p′* du DDE. L'analyse d'échantillons prélevés parmi la population civile de Delhi montre une teneur significativement plus élevée en α- et β-HCH et en composés du groupe DDT que dans les fragments de tissu adipeux provenant de personnes appartenant au personnel militaire vivant dans d'autres régions de l'Inde. On ne relève aucune différence importante entre les taux de l'isomère *p,p′* du DDD et de la dieldrine dans la popu-

lation de Delhi et celle d'autres régions du pays. En revanche, le tissu adipeux des habitants de la région de Delhi contient moins de dieldrine que celui de certaines populations des Etats-Unis d'Amérique, mais sa teneur en HCH et en composés du groupe DDT est plus élevée. A Delhi et dans d'autres régions de l'Inde, le DDE intervient pour 42% et 52% respectivement dans l'ensemble des substances du groupe DDT. Cette proportion est très nettement inférieure à celle que l'on observe aux Etats-Unis (62%); les Indiens, consommant moins de viande, accumulent relativement moins de DDE que les populations d'autres pays. Il est possible que les habitants de l'Inde soient exposés à de plus fortes concentrations de DDD; les Indiens de Delhi sont certainement exposés à de plus fortes concentrations de DDT et d'HCH que la population d'autres pays.

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Dehydrogenation: A Previously Unreported Pathway of Lindane Metabolism in Mammals

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This study presents evidence for the dehydrogenation of lindane by a hepatic microsomal mixed-function oxidase system. Preliminary investigation established that the incubation of lindane with rat liver homogenates produces a chlorinated, nonpolar compound identified as hexachlorocyclohexene. Differential centrifugation resulted in the sedimentation of most of the dehydrogenase activity in the microsomal fraction. Optimum *in vitro* assay conditions were established and it was found that the dehydrogenase system required molecular oxygen and reduced pyridine nucleotide coenzyme for maximum activity. Inhibition by SKF 525-A and CO suggested that the enzyme was cytochrome P-450 dependent. Lack of inhibition by cyanide indicated that the cytochrome b_5 desaturase system was probably not involved. Pretreatment of rats with DDT, which stimulates lindane metabolism, also induced significantly higher dehydrogenase activity. Both the *in vivo* and *in vitro* metabolism of hexachlorocyclohexene produced previously identified lindane metabolites. The existence of a cytochrome P-450 dependent mixed-function oxidase which catalyzes the dehydrogenation of lindane has not previously been reported and may be of importance in the metabolism of other xenobiotics.

It is generally accepted that dehydrochlorination of lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane, γ-HCH[1]) to γ-2,3,4,5,6-pentachlorocyclohex-1-ene (γ-PCCH) is the first step in the metabolism of this organochlorine insecticide by both plants (1, 2) and animals (3-14). In 1965 Grover and Sims showed that rats metabolize both lindane and γ-PCCH to 2,3,5 and 2,4,5-trichlorophenol which are excreted in the urine as free phenols, sulfates, and glucuronic acid conjugates (14). Because the metabolites identified in their study resembled those from the degradation of 1,2,4-trichlorobenzene in rabbits (15), the authors speculated that lindane metabolism proceeds via γ-PCCH as an intermediate step to 1,2,4-trichlorobenzene. Currently, most of the published schemes of lindane metabolism are based on this work by Grover and Sims (16-18) and indicate that lindane is metabolized almost exclusively by dehydrochlorination through the intermediate metabolite γ-PCCH. Nevertheless, recent identification of some previously unreported lindane metabolites in flies (11), and the urine of both rats (19) and rabbits (20) led to speculation that there might be important metabolic path-

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[1] The following abbreviations are used: γ-HCH, γ-1,2,3,4,5,6-hexachlorocyclohexane; γ-PCCH, γ-2,-3,4,5,6-pentachlorocyclohex-1-ene; HCCH, Hexachlorocyclohexene; PCCOL, 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol; G6-P, Glucose-6-phosphate; GSH, glutathione; DEGS, diethyleneglycosuccinate.

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ways other than the accepted dehydrochlorination of lindane through γ-PCCH. This study presents evidence to support the existence of a different initial metabolic pathway, specifically the dehydrogenation of lindane to the intermediate metabolite, hexachlorocyclohexene (HCCH).

MATERIAL AND METHODS

Animals

Weanling female Sherman and Sprague–Dawley rats were used in this study. The animals were housed in individual metabolism cages and when treated received po injections of peanut oil solutions of the compounds investigated. Since the rate of lindane metabolism *in vitro* is very low in livers from normal rats, optimum assay conditions were established using liver preparations from rats treated po with 2 mg DDT/day for 2 wk. All animals were sacrificed by decapitation to avoid any complications due to anesthetics.

Chemicals

All pesticides and pesticide metabolites except 1,2-dichlorochlordene, which was provided by Velsicol Chemical Corporation, Chicago, Ill. and those synthesized or isolated from rat urine, were obtained from the Pesticides and Toxic Substances Effects Laboratory, Analytical Reference Standards Repository, Research Triangle Park, N. C. Hexachlorocyclohexene (HCCH) was synthesized by photochlorination of *o*-dichlorobenzene (21). It was isolated by elution with hexane from a silicic acid column. Comparison of synthetic HCCH with the metabolite extracted from liver incubation mixtures, employing lindane as the substrate, demonstrated that the compounds are identical. The mass spectra of the compounds have the same molecular ion, chlorine isotope distribution, and fragmentation pattern. While complete characterization of this intermediate metabolite will be reported in another publication, the salient ions and their intensities include ($M^{+\cdot}$286 m/e (3%), $M^{+\cdot} - Cl$ 251 m/e (6%), $M^{+\cdot} - [Cl + HCl]$ 251 m/e (83%), $M^{+\cdot} - C_2H_2Cl_2$ 190 m/e (2%), $M^{+\cdot} - [Cl_2 + HCl]$ 180 m/e (74%)). The compounds have identical retention times on the different glc packing materials used in this study and both elute similarly from a silicic acid column. The lindane metabolite 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol (PCCOL) was isolated from urine and identified previously (19). Dithiothreitol was obtained from Calbiochem, San Diego, Cal. Nicotinamide, glucose-6-phosphate (G6-P), glutathione (GSH), cysteine, G6-P dehydrogenase, NAD⁺, NADH, NADP⁺, NADPH, and Trizma preset pH crystals for Tris-HCl buffer were purchased from Sigma Chemical Company, St. Louis, Mo. SKF 525-A was purchased from Smith, Kline, and French Laboratories, Philadelphia, Pa. EDTA was obtained from Fisher Scientific Company, Pittsburgh, Pa. 1.5% OV-17 + 1.95% QF-1, on 80/100 mesh Gas Chrom Q was obtained from Applied Science Laboratories Inc., State College, Pa. Diethyleneglycosuccinate (DEGS) and Gas Chrom Q were obtained from Supelco, Inc., Bellefonte, Pa. Gas chromatographic grade solvents were used for all extractions.

Tissue Preparation

Livers were quickly removed, weighed, and a 1 g portion was homogenized with 5 ml of 0.1 *M* Tris-HCl buffer, pH 7.5, in a Potter–Elvehjem homogenizer with a motor driven Teflon pestle. The homogenization was carried out at 4°C for 1 min. The homogenate was centrifuged at 9000*g* for 20 min in a refrigerated Sorval superspeed centrifuge. The reddish supernatant was used for most of the assays conducted in this study. When microsomes were isolated, the 9000*g* supernatant was centrifuged at 105,000*g* for 75 min in a refrigerated Beckman Model L-2 Ultracentrifuge. The resulting glassy-red pellet was well drained and homogenized by hand into a volume of 0.1 *M* Tris-HCl buffer equal to the volume of the solution that was centrifuged at 105,000*g*. Protein concentration was determined by the Lowry method (22).

Enzyme Assays

The standard incubation mixture of 1 ml of the 9000*g* supernatant (or resuspended microsomes) plus 2 ml of an aqueous mixture containing 160 μmol of Tris-HCl buffer, pH 7.5, 60 μmol of nicotinamide, 2 μmol of NADP$^+$, 10 μmol of G6-P, 0.4 units of G6-P dehydrogenase, and 0.34 μmoles of lindane dissolved in 0.1 ml of acetone. The mixture, in a 10 ml Erlenmeyer flask, was incubated for 30 min in a Dubnoff Metabolic Shaking Incubator at 150 oscillations/min and 37°C. The reaction was terminated by the addition of 1.5 ml of 1 *N* HCl to the incubation mixture. In other experiments, acetone solutions of γ-HCCH, cis- and trans-chlordane, and α-, β- and δ-hexachlorocyclohexane were incubated as substrates in place of lindane. In the determination of V_{max} and K_m values, a 20 min incubation time was used.

Extractions

The incubation mixture was transferred to a 15 × 125 mm screwcapped test tube containing approximately 1 cm of NaCl in the bottom of the tube. The incubation flask was rinsed with 1.5 ml of 1 *N* HCl followed by 5 ml of benzene. Both the HCl and the benzene were transferred to the test tube containing the incubation mixture. The combined mixture in the test tube was then shaken vigorously on a vortex mixer for 1 min. The resulting emulsion was broken by centrifugation at 3000 rpm for 3–5 min and the benzene was pipetted off. The extraction was repeated once more and the combined extracts were analyzed by gas chromatography.

Chromatography

Metabolites were assayed on a Micro-Tek MT-220 gas chromatograph equipped with an electron-capture detector and a Coulson electrolytic conductivity detector. The columns used for analysis were 6 ft × 0.25 in. glass U-tubes packed with either 1.5% OV-17 + 1.95% QF-1, on 80/100 mesh Gas Chrom Q or 5% DEGS on 80/100 mesh Gas Chrom Q and both were isothermally maintained at 170°C. The flow of nitrogen carrier gas was maintained at 50 cm^3/min.

Statistics

Data were analyzed by the use of Student's *t* test.

RESULTS

In Vitro Metabolism of Lindane in the Rat

Preliminary investigation established that the incubation of lindane with rat liver homogenates produced a chlorinated, nonpolar metabolite which had an identical mass spectra and identical GLC retention time to synthetically prepared hexachlorocyclohexene. The metabolite also eluted from silicic acid similarly to hexachlorocyclohexene. Consequently, the *in vitro* dehydrogenase activities of both control and induced animals were investigated and compared.

Intracellular Distribution

Differential centrifugation resulted in the sedimentation of the highest specific dehydrogenase activity in the microsomal (105,000*g*) pellet as indicated in Table 1.

Optimum Assay Conditions

The 9000*g* supernatant from the centrifugation of rat liver homogenates was used to establish optimum *in vitro* assay conditions for the dehydrogenase system.

Effect of time. Figure 1 indicates that the amount of HCCH formed during the dehydrogenation of lindane was reasonably linear up to 30 min and leveled off thereafter.

TABLE 1

Microsomal Dehydrogenase in Subcellular Fractions Prepared by Differential Centrifugation from Homogenates of Rat Liver

Fraction[a]	Total protein (mg)	Total activity (µg HCCH/30 min)	Relative sp act[b]
Homogenate	28	0.299	0.16
Mitochondrial pellet	24	0.069	0.04
9000*g* Supernatant	23	0.496	0.32
Microsomal pellet	6	0.430	1.00
105,000*g* Supernatant	17	0.022	0.01

[a] Preparation of subcellular fractions and incubation conditions as described in Methods.

[b] Relative sp act = (Total activity of fraction/fraction total protein)/(Total activity of Microsomal Pellet/Microsomal Pellet Protein).

Effect of pH. The optimum pH for the dehydrogenase system was 7.5 as indicated in Fig. 2.

Effect of protein concentration. As illustrated in Fig. 3, dehydrogenase activity was proportional to microsomal protein concentration at the level routinely employed in the assay (0.2*g* wet liver wt or 23 mg of protein in the aliquot of the 9000*g* supernatant taken for analysis).

Effect of substrate concentration. The apparent Michaelis constants (K_m) for the dehydrogenase systems from both control

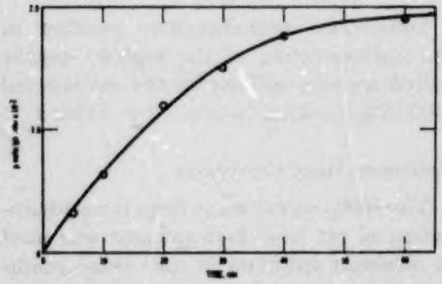

FIG. 1. *Effect of time (minutes) on the* in vitro *metabolism of lindane to hexachlorocyclohexene (µmole/gram liver × 10^{-2}). Incubation conditions as described in Methods. Each value is the mean of two determinations.*

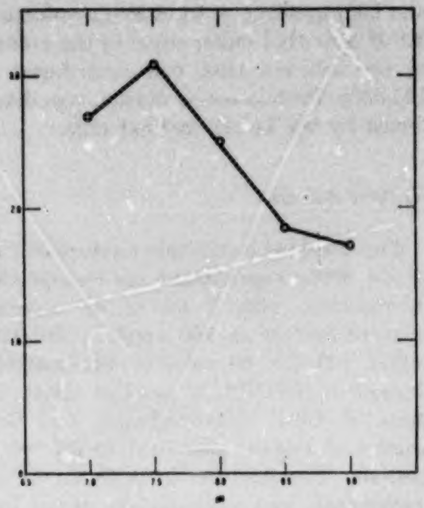

FIG. 2. *Effect of pH on the* in vitro *metabolism of lindane to hexachlorocyclohexene (µmole/gram liver/hour × 10^{-2}). Incubation conditions as described in Methods. Every value represents the mean of two determinations on each of the liver preparations from two rats.*

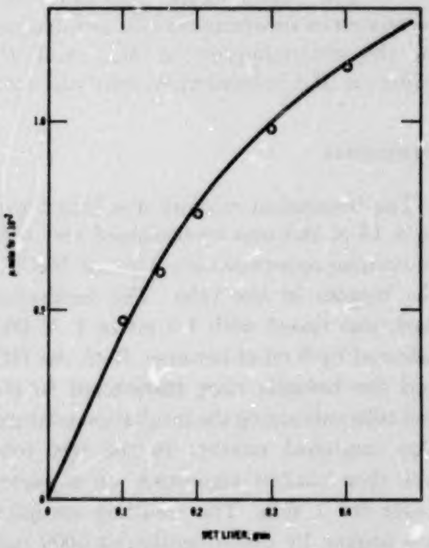

FIG. 3. *Effect of protein concentration (grams wet liver) on the* in vitro *metabolism of lindane to hexachlorocyclohexene (µmole/hour × 10^{-2}). Incubation conditions as described in Methods. Each value is the mean of two determinations.*

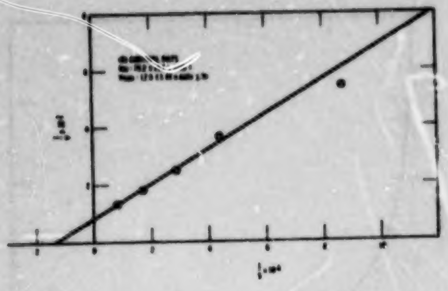

FIG. 4. *Lineweaver–Burk plots for the* in vitro *metabolism of lindane to hexachlorocyclohexene by control rats. S = substrate added (mole/liter); V = product formed (µmole/gram/hour). Incubation conditions as described in Methods. Every value is the mean of two determinations on each of the liver preparations from three rats.*

(Fig. 4) and DDT-treated rats (Fig. 5) were determined from the double reciprocal plots of Lineweaver and Burk (23). The apparent mean K_m ± SE for the DDT and control animals, 20.0 ± 0.60 and 79.2 ± 10.5 µmol of lindane per liter, respectively, were significantly different at $P < 0.01$. The lower K_m value of the DDT treated rats could be the result of a higher affinity between the substrate lindane and the enzyme system, the induction of a new enzyme or perhaps a "deinhibition" of the dehydrogenase enzyme system in these animals. The maximal velocity (V_{max} ± SE) of the control animals (12.5 ± 1.4 nmol HCCH formed/g/h) was increased more than threefold (41.2 ± 10.7 nmol HCCH formed/g/h) by the DDT treatment.

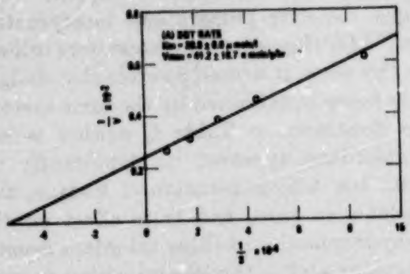

FIG. 5. *Lineweaver–Burk plots for the* in vitro *metabolism of lindane to hexachlorocyclohexene by DDT treated rats. S = substrate added (mole/liter); V = product formed (µmole/gram/hour). Incubation conditions as described in Methods. Every value is the mean of two determinations on each of the liver preparations from five rats.*

TABLE 2

The Coenzy[illegible] Requirements of the Dehydrogenase [illegible]m from Rat Liver

Incubation medium[a]	Relative dehydrogenase activity
Complete 9000g supernatant	100
–dehydrogenase enzymes	0
–dehydrogenase enzymes plus enzymes heated at 100°C, 2 min	0
–Nicotinamide (60 µmol)	62
–Nicotinamide (60 µmol) mi[illegible] NADPH generating system	6
–NADPH generating system	44
–NADPH generating system plus NADH (2 µmol)	90
–NADPH generating system plus NADPH (2 µmol)	90
–NADPH generating system plus NAD (2 µmol)	78
–NADPH generating system plus NADP (2 µmol)	67
Complete microsomal preparation	100
–NADPH generating system plus NADH (2 µmol)	29
–NADPH generating system plus NADH (10 µmol)	40
–NADPH generating system plus NADH (20 µmol)	48
–NADPH generating system plus NADPH (2 µmol)	48
–NADPH generating system plus NADPH (10 µmol)	115
–NADPH generating system plus NADPH (20 µmol)	118

[a] "Complete" incubation medium and conditions as described in Methods.

Requirements of the dehydrogenase system. As shown in Table 2, the dehydrogenase system required reduced pyridine nucleotide for complete activity and NADPH appeared to be preferred over NADH. While both NADH and NADPH provided almost maximal activity with the 9000*g* supernatant, NADPH supported twofold more activity in the microsomal fraction. The data further suggested that the increased activity provided by NADH in the 9000*g* supernatant was due to the presence of endogenous $NADP^+$ and an NADPH-generating system. Nicotinamide was also required, probably because it protects against NADP degradation (24–29).

Data in Table 3 show that the dehydrogenase system was not affected by addition of 4 mmol EDTA or $MgCl_2$ up to 16 µmol. Cyanide, which is known to inhibit other

TABLE 3

Inhibition of Dehydrogenase Activity

Incubation medium[a]	Relative dehydrogenase activity
Complete 9000*g* supernatant	100
+Mg Cl_2 (16 μmol)	100
+EDTA (4 mmol)	100
+NaCN (5 μmol)	106
+SKF 525-A (1 mmol)	18
+SKF 525-A (100 μmol)	63
+O_2 (bubbled gently 1 min)	100
+N_2 (bubbled gently 1 min)	0
+CO (bubbled gently 1 min)	0

[a] "Complete" incubation medium and conditions as described in Methods.

dehydrogenases (30, 31), had little effect on the dehydrogenase system that metabolizes lindane. Though not included in Table 3, glutathione, cysteine, dithiothreitol, and EDTA, which have been used to stabilize or enhance the activity of various dehydrogenases (32–34) had no effect on the dehydrogenation of lindane. Results from Table 3 indicate that SKF 525-A, which by binding to microsomes and soluble cytochrome P-450 greatly inhibits interactions of substrates with cytochrome P-450 (35), was a potent inhibitor of the dehydrogenase activity. Furthermore, dehydrogenase activity was completely inhibited following a brief (1 min) exposure to either CO or N_2. When N_2 was used, lindane was metabolized by a different route to a different metabolite, which was tentatively identified by mass spectroscopy as γ-3,4,5,6-tetrachlorocyclohex-l-ene and the enzyme system appeared to be similar to that reported by Walker (36). Various CO/O_2 ratios were used to determine *K*, the Warburg partition coefficient (37), which is the CO/O_2 ratio required for 50% inhibition of the dehydrogenase system. The ratio was found to be 1.8 (Fig. 6) which is in line with CO/O_2 ratios required for 50% inhibition of various other hepatic microsomal mixed function oxidases (38).

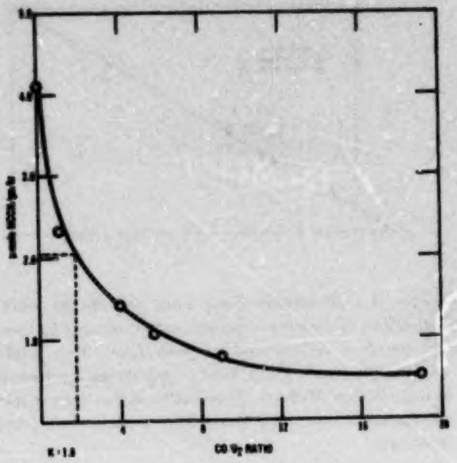

FIG. 6. *Effect of varying the CO/O_2 atmosphere on the* in vitro *metabolism of lindane to hexachlorocyclohexene (μmole/gram liver/hour). The Warburg partition coefficient K = the CO/O_2 ratio required for 50% inhibition of the metabolism of lindane to hexachlorocyclohexene. Incubation conditions as described in Methods. Each value is the mean of two determinations.*

Specificity of the Dehydrogenase System

Both γ-chlordane (1-exo-2-endo-4,5,6,7,-8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene) and α-chlordane (1-exo-2-exo-4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene) are metabolized *in vitro* by dehydrogenation to the intermediate 1,2,-dichlorochlordene (39). Since rat liver preparations incorporating an NADPH-generating system were utilized in this work, it seemed possible that lindane was being metabolized by the same system. As illustrated in Table 4, neither γ- nor α-chlordane appeared to significantly inhibit the dehydrogenation of lindane, and lindane, in turn, had little effect on the dehydrogenation of either chlordane isomer. Previous work in this laboratory has demonstrated that when separate groups of rats are administered one of four configurational isomers of hexachlorocyclohexane (α-, β, γ-, or δ-HCH) only the group receiving the γ-HCH excreted PCCOL, 2,3,4,6-, and 2,3,4,5-tetrachlorophenol (40). Since these

TABLE 4

Specificity of the Dehydrogenase System from Rat Liver

Substrates utilized[a]	Relative dehydrogenase activity	
	Hexachlorocyclohexene	Dichlorochlordene
γ-HCH	100	
α-chlordane		100
α-chlordane + γHCH	87	89
γ-chlordane		100
γ-chlordane + γHCH	83	94
α-HCH + γHCH	84	
β-HCH + γHCH	88	
δ-HCH + γHCH	54	

[a] One hundred micrograms of each substrate was used in the standard incubation mixture described in Methods.

metabolites could result from a pathway involving a stereospecific dehydrogenase, the metabolism of combinations of lindane (γ-HCH) with the α-, β-, and δ-isomers were compared to that of lindane alone for competitive inhibition of the dehydrogenase system. Only the δ-HCH significantly inhibited the *in vitro* dehydrogenation of lindane indicating that the dehydrogenase exhibited some stereospecificity (Table 4).

In Vivo Metabolism of Hexachlorocyclohexene

Rats administered synthetic HCCH, excreted all previously identified lindane metabolites for which adequate analytical procedures have been developed (Table 5). Moreover, the total metabolism of HCCH to chlorophenols and PCCOL took place almost twice as readily as that of lindane. However, while the quantities of 2,3,5-, 2,4,5-trichlorophenol and 2,3,4,6-tetrachlorophenol excreted by rats receiving HCCH were significantly greater than that excreted by lindane treated rats, the lindane treated animals excreted more 2,4,6-trichlorophenol. No significant differences in the excretion of 2,3,4,5-tetrachlorophenol or PCCOL by the two groups were noted.

In Vitro Metabolism of Hexachlorocyclohexene

The *in vitro* incubation of HCCH with liver preparations produced PCCOL and all five of the chlorophenols recovered from the urine of rats treated with either lindane or hexachlorocyclohexene (Table 6). Pretreatment of the rats with DDT significantly induced the metabolism of HCCH to all of these metabolites. The amount of PCCOL formed was at least five times greater than any of the other metabolites formed *in vitro*, whereas *in vivo* the reverse was true. This could indicate that PCCOL was metabolized further *in vivo* or it could be explained by the fact that optimum incubation conditions were not employed for the other enzymes responsible for HCCH metabolism *in vitro*.

DISCUSSION

Currently, it is accepted that dehydrochlorination of lindane to γ-PCCH is the critical initial step in the metabolism of this organochlorine insecticide by mammals (16–18). However, it is difficult to account for the chemical structure of some previously identified lindane metabolites by a metabolic pathway in which dehydrochlorination is the first step. For example, it was necessary to hypothesize an allylic oxidation with a double bond shift to account for the formation of PCCOL from γ-PCCH in the rat (18), and no attempt has been made to account for the metabolism of lindane to pentachlorophenol in rabbits. Furthermore, a previous report from this laboratory indicated that γ-PCCH is metabolized by the rat to only 2,4,5- plus a trace of 2,3,5-trichlorophenol and not to PCCOL or to the other chlorophenols (40). On the other hand, HCCH is metabolized by the rat to PCCOL and all of the chlorophenols produced from lindane (Tables 5

TABLE 5

Comparative in vivo Metabolism of Lindane and HCCH

Compound administered[a]	Avarage μg metabolite excreted/24 H ± SE[b]					
	246-TCP[c]	235-TCP[d]	245-TCP[e]	2346-TTCP[f]	2345-TTCP[g]	PCCOL[h]
Lindane	51.1 ± 8.27	8.43 ± 0.981	14.7 ± 3.22	27.1 ± 4.06	4.32 ± 2.82	0.25 ± 0.067
HCCH	20.6 ± 4.10[i]	33.6 ± 2.50[i]	50.1 ± 4.24[i]	86.8 ± 11.2[i]	5.39 ± 0.990	0.48 ± 0.120

[a] Two milligrams of the respective compound/0.1 ml peanut oil was administered to each rat.

[b] Excretion values represent the mean of six animals ± SE.

[c] 2,4,6-Trichlorophenol.

[d] 2,3,5-Trichlorophenol.

[e] 2,4,5-Trichlorophenol.

[f] 2,3,4,6-Tetrachlorophenol.

[g] 2,3,4,5-Tetrachlorophenol.

[h] 2,3,4,5,6-Pentachloro-2-cyclohexen-1-ol.

[i] $P < 0.05$.

and 6). A pathway involving the initial dehydrogenation of lindane to HCCH should significantly simplify attempts to account for known lindane metabolites in mammals.

Apparently, this is the first report of a cytochrome P-450 dependent mixed function oxidase which requires molecular oxygen and reduced pyridine nucleotide coenzyme, and catalyzes the dehydrogenation of a pesticide. Although other microsomal dehydrogenases have been reported (41–46), these do not require cytochrome P-450 or reduced pyridine nucleotide coenzyme. Though both systems require molecular oxygen and reduced pyridine nucleotide coenzyme, the mixed-function oxidase responsible for lindane dehydrogenation differs from the fatty acid desaturase described by Oshino (31) in that the dehydrogenation of lindane is cytochrome P-450 dependent and not sensitive to cyanide inhibition, whereas the opposite holds true for the desaturase system.

It is possible that the dehydrogenase system metabolizing lindane is the same

TABLE 6

In Vitro Metabolism of Hexachlorocyclohexene by Rat Liver[a]

Animal pretreatment[b]	Average μg metabolite produced/30 min					
	246-TCP[c]	235-TCP[d]	245-TCP[e]	2346-TTCP[f]	2345-TTCP[g]	PCCOL[h]
Control	2.2 ± 0.12	0.74 ± 0.10	1.3 ± 0.07	4.3 ± 0.40	0.50 ± 0.06	25 ± 3.5
DDT	4.8 ± 0.05[i]	2.1 ± 0.18[i]	6.0 ± 0.23[i]	11 ± 0.36[i]	0.92 ± 0.04[i]	40 ± 2.9[i]

[a] Four hundred micrograms of HCCH was used in the standard incubation mixture described in Methods.

[b] Pretreatment as described in Methods.

[c] 2,4,6-Trichlorophenol.

[d] 2,3,5-Trichlorophenol.

[e] 2,4,5-Trichlorophenol.

[f] 2,3,4,6-Tetrachlorophenol.

[g] 2,3,4,5-Tetrachlorophenol.

[h] 2,3,4,5,6-Pentachloro-2-cyclohexen-1-ol.

[i] $P < 0.05$.

as that which converts α- and γ-chlordane to 1,2-dichlorochlordene (39) or that which dehydrogenates pyrrolizidine alkaloids (47). However, the intracellular distribution and cofactor requirements of the enzyme which dehydrogenates chlordane have not been reported. Furthermore, saturating levels of either α- or γ-chlordane fail to significantly inhibit the dehydrogenation of lindane *in vitro*, and lindane has little effect on the dehydrogenation of either chlordane isomer (Table 4). This suggests that either different dehydrogenases or different binding proteins for the same dehydrogenase may be involved here. Similarities between the microsomal enzyme catalyzing the dehydrogenation of lindane and that reported to convert pyrrolizidine alkaloids to dihydropyrrolizine derivatives include a requirement for molecular oxygen, reduced NADP+, and inhibition by CO and SKF 525-A together with induction by DDT pretreatment (47). The authors of that study indicate that a new type of microsomal reaction need not be hypothesized. They propose a mechanism for the dehydrogenation of pyrrolizidine alkaloids which might involve initial C-hydroxylation or N-demethylation followed by a spontaneous sequence of nonenzymatic reactions. Such a mechanism, however, is not applicable to the metabolism of lindane. Moreover, the fact that the dehydrogenation of lindane apparently does involve a previously unreported type of microsomal reaction leads to speculation that the pyrrolizidine alkaloids and lindane may actually be metabolized by the same enzyme system. The metabolic dehydrogenation of these alkaloids produces toxic pyrrole derivatives and the detoxification of at least two pesticides, lindane and chlordane, may involve the same pathway. This enzyme system should be further characterized to determine its possible implication in the toxicology and metabolism of other xenobiotics.

Hamilton has suggested that the oxenoid mechanism may be involved in the dehydrogenation catalyzed by microsomal enzymes (48). On the basis of analogies with carbene (49) and nitrene (50) reactions, in addition to model reaction studies, Hamilton proposed that the equivalent of a free oxygen atom, with its six outershell electrons, abstracts two hydrogens to give water and the alkene directly (Fig. 7).

The oxenoid reagent $E - X = O$ is generated from O_2 and a group on the enzyme by reduction. Hamilton has speculated that the structure of the oxenoid reagent could be a peracid or a peramide formed from a carboxyl or amide group and the superoxide ion $O_2^{-}\cdot$, or Fe (III)$\cdot O_2^{-}\cdot$. In numerous examples of the oxidative formation of nonactivated double bonds (conjugated at the most to a second double bond), the removal of two hydrogens occurs by a *cis* elimination and is consistent with the oxenoid mechanism (51–62). This list includes double bond formation in steroid rings, steroid side chains, and fatty acids.

It is proposed that the dehydrogenation of lindane by a hepatic, cytochrome P-450 dependent mixed function oxidase represents an important initial step in the metabolic pathway leading to the detoxification of this pesticide by mammals.

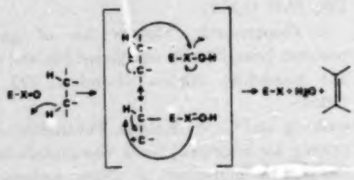

FIG. 7. *Possible mechanism for the dehydrogenation of lindane by microsomal cyt. P-450. E−X=O represents the oxenoid reagent, generated from O₂ and a group on the enzyme, which abstracts two hydrogens from a substrate such as lindane to give water and the alkene directly.*

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Effects of DDVP Aerosols on Blood Cholinesterase of Fogging Machine Operators

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0,0-Dimethyl-2,2-dichlorovinyl phosphate (DDVP) has been found to be very effective in the control of the cigarette beetle in tobacco warehouses.[1] One method of application is to blow the volatile insecticide into the building from a Todd Insecticidal Fog Applicator (TIFA) placed outside the warehouse. Although the toxicity of DDVP has been studied,[2] no investigation has been made of the effect of the above operations on the health of the men who operate these machines. Such studies are presented in this report. Since DDVP is an organic phosphate insecticide and readily inhibits blood or plasma cholinesterase,[3] it was possible to ascertain the possible exposure of these men to the insecticide by measurement of the level of these enzymes in the blood. In addition, each man was given a periodic physical examination in order to determine whether symptoms characteristic of poisoning with organic phosphate compounds had appeared.

Methods

The 12 men studied in this survey each worked a total of 45 hours a week for four months applying insecticides, except for Nos. 8 and 11, who operated the fogging machines for only two months. Work with DDVP was begun just after May 7, 1958. These workers applied 4% DDVP for eight hours a day on Monday and Thursday and a mixture of 3% lindane and 2% DDT in deodorized oil base on Tuesday, Wednesday, and Friday. The rate of spraying of both formulations was approximately 9 gal. per hour. Three of the men (Nos. 1, 2, and 7) formulated the DDVP insecticide by mixing 1 lb. of technical DDVP with 1 lb. of ethylene dichloride and 3 gal. of Bayer D. These persons wore protective rubber clothing, rubber gloves, and industrial-type gas masks during the formulating procedure. The men did not wear protective clothing or masks while operating the fogging machines but were instructed to stay on the windward side of the equipment during operation in order to minimize exposure to the DDVP aerosol.

The workers probably received their greatest exposure to the insecticide when the machine was started up or stopped, because at that time they had to get closer to the point of application of the aerosol. Also, since it was not always possible to stay on the windward side of the equipment, exposure was sometimes increased by this circumstance. Occasionally a man would enter the warehouse for three to five minutes after the DDVP was applied. None of the men changed clothing before leaving the plant.

Each month the men were given a physical examination, and at the same time a blood sample was taken for cholinesterase assay by the Michel procedure.[4]

Results and Conclusions

The physical examination gave no evidence of neurological symptoms which might be associated with the inhibition of cholinesterase by DDVP or with poisoning with DDT or lindane. The cholinesterase levels in the blood of these applicators were not decreased during the period of exposure. The results indicate that no physiologically significant exposure to DDVP took place.

Under the conditions of this study, the chlorinated hydrocarbons with which the men came in contact would not be expected to influence the levels of blood cholinesterases. The titer of plasma cholinesterase remains unchanged in men with very high occupational exposure to DDT.[4] It is true that the level of serum aliesterase is ele-

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vated in rats exposed to various chlorinated hydrocarbon insecticides, but the dosages used with the animals were high enough to produce clinical symptoms of poisoning.[5,6]

In some other studies with applicators who worked under different conditions and with organic phosphorus compounds different from those in the present survey, it was found that the cholinesterase levels of the blood of these spray operators gradually became lower as the spray season progressed.[7-10] Although the results are somewhat variable, it can be seen in the Table that this was not the case in the present study. It appears from these results that the procedure used for the application of DDVP presents no hazard from the insecticide to the men operating the Todd Insecticidal Fogging Applicator over a four-month period, providing these machines are properly used.

Summary

The levels of blood cholinesterases were studied in men who used Todd Insecticidal Fogging Machines to apply 4% 0,0-dimethyl-2,2-dichlorovinyl phosphate (DDVP) aerosols for 16 hours a week to tobacco warehouses. No decrease in the concentration of the plasma or red blood cell enzyme was found over a period of four months. It is concluded that this method of application of DDVP does not constitute a hazard to the health of these workers, providing the fogging machines are properly used.

Mr. Charles M. Sprinkle, Coordinator of Agricultural Research, R. J. Reynolds Tobacco Company, Winston-Salem, N. C., assisted in this study. The physical examinations were done by Dr. R. W. Bunn, Medical Director, R. J. Reynolds Tobacco Company. Miss Louise McLeod, Histopathology Technician of the Technical Development Laboratories, determined the plasma and red blood cell cholinesterase levels.

U. S. Department of Health, Education, and Welfare.

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Plasma and Red Blood Cell Cholinesterase Levels of DDVP Applicators

	Date Blood Sample Taken									
	May 7 *		June 4		July 7		Aug. 5		Sept. 5	
Applicator	R B C †	Plasma †	RBC †	Plasma †	RBC †	Plasma †	RBC †	Plasma †	RBC †	Plasma †
1	0.84	1.06	0.83	1.14	0.83	1.05	0.95	0.91	0.97	0.90
2	0.85	0.96	0.69	0.79	0.82	0.87	0.77	0.72	0.69	0.79
3	0.61	0.36	0.91	0.47	1.00	0.56	0.80	0.52	0.98	0.44
4	0.71	0.85	0.92	1.06	1.10	1.26	0.86	1.04	1.05	0.92
5	0.66	1.06	0.75	1.20	0.89	1.21	1.02	1.24	0.86	1.24
6	0.64	1.18	0.87	1.22	0.86	1.17	—	—	0.84	1.14
7	0.50	1.03	0.84	1.20	1.01	1.16	0.82	1.20	0.84	1.10
8	0.50	0.60	0.76	0.74	1.02	0.69	—	—	—	—
9	0.48	1.01	0.76	1.26	1.22	—	0.75	0.97	0.86	1.24
10	0.44	0.92	0.71	0.89	0.83	1.00	—	—	0.77	0.79
11	—	—	—	—	0.78 *	0.80 *	0.82	0.90	0.58	0.94
12	0.43	1.17	0.67	1.33	1.10	1.28	—	—	0.88	1.18

* Preexposure levels.
† Δ pH per hour per 0.02 ml. of packed cells or plasma.

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Measurement of Endrin Following Epidemics of Poisoning

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Food and Drug Administration
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Between 3 June and 15 July 1967, four explosive outbreaks of acute poisoning with the insecticide endrin occurred in Doha, Qatar and Hofuf, Saudi Arabia. They resulted in the hospitalization of 874 persons and the death of 26 persons. The outbreaks probably caused a lesser degree of poisoning in many others who recovered without seeking medical aid. A thorough epidemiological study reported by Weeks (1) revealed that flour in a lower deck of each of two ships had been contaminated by an emulsifiable concentrate of endrin carried on an upper deck of the same hold. The episodes were unrelated, but leakage in each instance probably occurred when the insecticide was unloaded by net. The flour, which was taken off later at different ports, led to poisoning when separate lots of it were made into bread and eaten.

This paper reports only our chemical studies concerned with the outbreaks. It describes the method used, and summarizes the chemical evidence that endrin from contaminated flour was, in fact, the cause of illness. The paper also presents available measurements pertaining to the distribution of endrin and its rate of loss in man.

Methods

The samples were collected in each country by consultants sent by the World Health Organization. An organophosphate insecticide was thought by local authorities to be the cause of the illness. A WHO team found an insecticide formulation (BHC) on a shelf in a bakery in Doha, Qatar. This insecticide formulation was also suspected as the probable contaminant. Selected benzene[1] extracts of bread and flour were analyzed by flame photometry and electron-capture gas-liquid chromatography for organophosphates and for chlorinated hydrocarbons. The findings were negative for organophosphates and γ-BHC in the flour and bread. The insecticide formulation was found to contain 11.75% γ-BHC (1,2,3,4,5,6-hexachlorocyclohexane) as the active ingredient. However, a compound in the bread and flour was isolated and identified as endrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo-endo-5,8-dimethanonaphthalene). This identification was confirmed

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[1] Pesticide quality benzene used.

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by electroconductometric gas-liquid chromatography, thin-layer and paper chromatography, infrared and NMR spectroscopy.

The experimental details are given in three parts: the extraction procedures, the gas chromatographic procedures, and the confirmational analysis.

Extraction. The bread samples were thoroughly ground in a mortar. These ground samples (150-875 mg) were quantitatively transferred to 15-ml glass-stoppered centrifuge tubes using 2 ml pesticide quality isopropanol and 4 ml pesticide quality benzene. They were extracted by shaking in a Scientific Products Delux Mixer.[2] After centrifugation, the isopropanol-benzene mixture was aspirated and transferred to another 15-ml centrifuge tube. Hexane-extracted distilled water was added. The benzene phase was separated from the aqueous isopropanol phase.

Flour samples were thoroughly mixed and extracted by the same procedure. The benzene extracts of the broad and flour samples were diluted or concentrated to appropriate volume and held for analysis by gas-liquid chromatography.

The plasma samples were extracted by the method of Dale et al. (2) and the urine samples by the method of Cueto and Biros (3).

A few samples of stomach wall, liver, and kidney taken at autopsy during the first outbreak in Doha, Qatar were the only tissues received. These samples were weighed (374-654 mg) and transferred to tissue grinders. Each sample was homogenized 3 times with 2 ml pesticide quality n-hexane. The hexane extracts were combined, centrifuged, concentrated to appropriate volumes, and held for analysis by gas-liquid chromatography.

Gas Chromatography. The gas chromatographic analyses were completed on a Micro Tek Model MT 220 instrument and a Varian Model 1525 equipped with tritium electron-capture detectors. The Micro Tek MT 220 instrument was operated with a 4' x 1/4" aluminum column packed with 3% Dow 220 on 70/80 mesh, acid-washed, DMCS-treated, Chromosorb G. The temperatures of the column, the inlet injection port, and the detector were 175°C, 200°C, and 200°C, respectively. The carrier gas was nitrogen at 60 psi with a flow rate of 60 cc/min. The detector was operated in the d.c. mode (15 v). The Varian instrument was operated with a 5' x 1/8" Al column packed with 3% OV-17 on 70/80 mesh, acid-washed, DMCS-treated, Chromosorb G. The temperatures of the column, the inlet injection port, and the detector were 175°C, 198°C, and 200°C, respectively. The carrier gas was nitrogen at 60 psi with a flow rate of 120 cc/min. The detector was operated at a fixed potential (d.c. 90 v). Other columns used were 3% QF-1 and 3% DEGS. Best results were achieved on the 3% OV-17 and 3% Dow 200 columns. The endrin standard gave three peaks on the 3% Dow 200 column with retention times of 9.0, 17.4, and 20.2 minutes, respectively. This same pattern was observed in the sample extracts. Possible chemical degradation of the compound on the column might account for the presence of three peaks. On 3% OV-17 one peak was observed at 14.5 minutes.

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[2] Use of trade names is for identification purposes only, and does not constitute endorsement by the Public Health Service, or the U.S. Department of Health, Education, and Welfare.

Confirmational Analysis. The identification of the contaminant (endrin) was confirmed by subjecting flour extracts to electroconductometric gas-liquid, thin-layer, and paper chromatography. A Micro Tek Model 2000 instrument equipped with a Coulson Electrolytic Conductivity Detector operated in the oxidative mode for chloride determination was used. The column and other operating parameters were the same as those with the Micro Tek MT-220 instrument. The observed retention times were identical to those mentioned earlier.

Five grams of flour were placed in a 15-ml centrifuge tube, 8 ml isopropanol or 6 ml acetone was added, and the mixtures were shaken vigorously. The tubes were then centrifuged at 2000 rpm for 5 minutes. Paper chromatography of spots from 100 ul aliquots of the supernatant liquid detected 50 ppm of endrin. Concentration of a 2.0-ml aliquot to 0.2 ml before spotting 100 ul increased the sensitivity to 5 ppm. The paper chromatograms were developed by two-phase ascending systems including:

System A, Immobile: 5% v/v paraffin oil in ether. Mobile: Acetone - water (3:1). Development time, 1 hour.

System B, Immobile: N,N'-dimethylformamide, 35% v/v in ether. Mobile: 2,2,4-Trimethylpentane. Development time, 45 minutes.

System C, Immobile: 5% v/v paraffin oil in ether. Mobile: Methyl alcohol-acetic acid-water (80:5:15). Development time, 1 hour.

The spots on the chromatograms were detected by Mitchell's spray for chloride compounds (0.4 g silver nitrate, 2.5 ml 2-phenoxyethanol, and 5 ml acetone, diluted to 50 ml with chloride-free water) (4). The R_f values corresponded to those for standard endrin and were 0.53, 0.89, and 0.45 for systems A, B, and C, respectively.

Thin-layer chromatography was accomplished on aluminum oxide and silica gel plates using pesticide quality n-hexane-ethyl ether (95:5) for development The R_f values for both samples and standard endrin were 0.35 on aluminum oxide and 0.31 on silica gel.

Verification that the flour sample extracts contained endrin was obtained by spotting the extracts on paper along with separate spots of standard solutions of endrin, dieldrin and a mixture of the two. These papers were then exposed to ultraviolet light for 1 hour. Two-phase chromatography of the papers by two systems of

TABLE 1

Endrin Concentrations Found in Various Samples From Three of the Four Outbreaks

Sample	Endrin concentration (ppm) First Doha outbreak	Third Doha outbreak	Hofuf outbreak
Bread, portions of which had been eaten by patients	48	1,339-1,807	243-537
Flour, used to make the same bread	INA[a]	2,153	3,367
Blood from patients	0.007-0.032	INA	0.008-0.027
Urine from patients	INA	INA	<0.004-0.007
Vomitus from patient	INA	INA	5.24
Tissues (autopsy) from:			
stomach wall	0.16	INA	INA
liver	0.685	INA	INA
kidney	0.116	INA	INA

[a] INA: Information not available.

TABLE 2

Concentration of Endrin in the Urine and Plasma Collected from Acutely Poisoned Patients on the Day of Onset

Patient's initials	Interval from ingestion to symptoms (hr)	Concentration of endrin (ppm) Plasma	Urine
H.N.	3.3	0.008	0.005
F.D.	1.5	0.011	<0.007
A.R.	1.5	0.027	<0.004

different polarity (4) verified that the flour extracts gave the same decomposition products upon exposure to U.V. light as were given by standard endrin.

Our results were confirmed independently at the pesticide laboratory at Perrine, Florida by three separate methods. Portions of flour, bread, and other samples were subjected to thin-layer chromatography, infrared spectrophotometry, and nuclear magnetic resonance spectrometry. The thin-layer chromatograms, IR spectra, and NMR spectra of the samples were compared with the thin-layer chromatograms, IR spectra and NMR spectra of pure endrin and of pure endrin that had been subjected to weathering. Virtually all spots on sample chromatograms were accounted for on the chromatograms of weathered endrin. All bands on the IR spectra of the samples were accounted for on the IR spectra of the weathered endrin also, as were the peaks on the NMR spectra. There was no doubt that the chromatograms and the spectra of the samples were those of endrin or its degradation products.

Results and Discussion

The flour used for making bread was not necessarily the most contaminated. The concentration of endrin in cloth from a stained area of one sack was 194,000 ppm; the concentration in flour from directly beneath the stain was 28,070 ppm; and the concentration in flour from deep within the sack was 4,460 ppm. As shown in Table I, the highest concentration of endrin found in flour known to have been used for making bread eaten by a patient was 3,367 ppm. The lowest concentration of endrin in bread known to have caused poisoning in one of the four outbreaks was 48 ppm. The lowest comparable value of 176 ppm was reported earlier by Coble *et al*. (5). Table I shows that endrin was measured not only in flour, bread, and vomitus, but also in blood, urine, and tissues from patients. Endrin is not found in comparable samples from the general population or even from workers who manufacture and formulate the insecticide (6).

We received samples of urine and plasma which had been obtained from three acutely poisoned patients on the day of onset of symptoms. Table II shows that endrin was more regularly detected in the plasma and that the concentration was higher than in the urine. The 14 late samples that we received were uniformly negative for endrin. By contrast, dieldrin is easily measured in the blood of people in the general population (2) and was still elevated 14 months after accidental ingestion leading to poisoning (7).

Dieldrin, p,p'-DDT, o,p'-DDT, p,p'-DDE, o,p'-DDE, and BHC were detected in the blood, urine, and tissue (stomach wall). The levels were no greater than those commonly found in the general population, and these compounds were entirely unrelated to the illness.

The chemical evidence presented clearly indicates that endrin was in fact the contaminant that caused the illness and deaths in the four outbreaks of poisoning in Doha, Qatar and Hofuf, Saudi Arabia. Endrin was detected in the blood and urine of patients, and in tissues taken at autopsy. The concentrations in the blood were similar to those found by Coble *et al*. (5). Samples of blood and urine taken 29-31 days after the outbreak were uniformly negative for endrin.

Acknowledgment

Infrared and NMR spectral analysis, electron-capture gas-liquid chromatography and thin-layer chromatography were completed as further confirmational analysis by the Pesticide Research Laboratory, Pesticide Program, Perrine, Florida.

Dr. Wayland J. Hayes, Jr., formerly with the PHS Pesticides Program, gave advice and guidance throughout the study. Dr. Hayes is now with the Department of Biochemistry, Vanderbilt University, Nashville, Tennessee.

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Effects of Dieldrin or DDT *in vivo* on Alpha-Alanine, Gamma-Aminobutyrate, Glutamine, and Glutamate in Rat Brain. (28948)

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The biochemical mechanism by which mammals are poisoned with chlorinated hydrocarbon insecticides, such as 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo, exo-5,8-dimethano-naphthalene (dieldrin) and 2,2-bis-(p-chlorophenyl)-1,1,1-trichloroethane (DDT), is unknown. Both of these insecticides produce convulsions in man (1-4) and experimental animals(5,6). The convulsions produced by dieldrin closely resemble the *grand mal* seizure of epilepsy. They occur without warning, are intermittent, and are clonicotonic in nature(3,4,6). On the other hand, animals exposed to a lethal dose of DDT show gradually increasing muscular tremor, which may last several hours and lead to convulsions that may be terminal(5). Symptoms observed in man have been similar to those noted in experimental animals(1,2). The marked difference in symptomatology between the two insecticides suggests a difference in their modes of action. Under these circumstances, any change noted with one substance and not with the other might reveal some clue as to a specific mode of action.

The free amino acid level of the brain is considerably higher than that of plasma and the pattern of the free amino acid pool is different from that of cerebral protein(7). A number of workers have investigated the effect of various convulsants on the levels of amino acids in brain, particularly the levels of glutamic acid, glutamine, and γ-aminobutyric acid(8-25). Further, it has been suggested that γ-aminobutyric acid may be a depressant neurohormone which may oppose the excitatory action of acetylcholine(26). As far as we are aware, no reports have appeared in the literature on the effects of dieldrin or DDT on these substances.

Therefore, a study was made of the levels of these amino acids in the brains of rats poisoned with DDT or dieldrin in an attempt to obtain some insight concerning the mode of action of these chlorinated compounds. In these experiments, the animals were dosed with large amounts of the toxicant to obtain severe poisoning. They were sacrificed when exhibiting pronounced symptoms but before moribund changes could begin.

Experimental section. Individually caged female albino rats of the Sherman strain, 4 to 4½ months of age and weighing 172-262 g, were used in all experiments. The insecticides were administered in peanut oil by stomach tube at the rate of 5 ml/kg body wt (160 mg/kg of dieldrin or 400 mg/kg of DDT). These dosage levels are approximately 4 times the oral LD_{50} dose of each insecticide(27). Control groups received peanut oil alone.

In preliminary experiments it was found that animals given dieldrin at this level survived 4 convulsions and died within 114 to 176 minutes after dosing, averaging 146 ± 23 (Standard Deviation) minutes. On the basis of these experiments, it was decided to sacrifice the animals during their fourth convulsion. Therefore, the dieldrin-poisoned animals were sacrificed within 72 to 109 minutes after dosing, averaging 89 ± 14 minutes. Onset of the first convulsion in the group occurred within 36 to 81 minutes after dosing, averaging 53 ± 15 minutes. In the case of the animals given DDT, preliminary experiments showed that death occurred within 200 to 470 minutes, averaging 394 ± 109 minutes. On the basis of these results, it was decided to sacrifice the DDT-poisoned animals after 120 minutes of visible body tremor. The onset of tremor was defined as a condition in which the animal's back was arched and the entire body was in tremor. This occurred within 100 to 180 minutes after dosing, averaging 131 ± 23 minutes.

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Control animals were sacrificed at the same time as corresponding rats in the experimental group. The rats were decapitated and the heads were dropped into liquid nitrogen within 4 seconds; the whole brain then was extracted essentially according to the procedure of Roberts and Frankel(28).

Amounts of extract equivalent to 20 mg of brain, fresh weight basis, were applied as 20 μl bands on Whatman No. 1 paper for the analysis of γ-aminobutyric acid, glutamic acid, and glutamine. Glutamic acid and γ-aminobutyric acid were separated at 25°C in phenol saturated with water and containing 0.025% 8-hydroxyquinoline. O-Phosphoryl ethanolamine runs in the same position as glutamic acid in this solvent but the amount present is so small, in relation to the large amount of dicarboxylic acid(29), that the interference in subsequent analysis was neglected. Glutamine was separated in a solvent containing 2-butanol—95% ethanol—6% ammonium hydroxide (100:25:35 v/v), as described by Roland and Gross(30). The ammonium hydroxide was prepared from concentrated ammonium hydroxide by diluting with 4 volumes of water. The mobility of glutamine in this solvent has not been described. After 72 hours of development at 25°C, this amide was found to run between the aspartic-glutamic acid and the serine-glycine-histidine spots, and its movement in relation to valine was 0.32 as compared with 0.12 for glutamic acid and 0.42-0.43 for serine-glycine-histidine. Recovery studies showed that the rate of hydrolysis of glutamine in this solvent was negligible. The quantity of amino acids present was determined from comparison with standards chromatographed at the same time, essentially according to the method described by Roberts and Frankel (31).

Alanine was separated by 2-dimensional chromatography. Twenty μl spots, equivalent to 40 mg of brain, fresh weight basis, were applied to 46 × 57 cm Whatman No. 1 filter paper, then chromatographed at 25°C in phenol-water followed by collidine-lutidine (1:1 v/v) saturated with water(32). It was not feasible to run more than 8 two-dimensional chromatograms at a time. Therefore, extracts of the brains of 4 control and 4 experimental animals were run simultaneously without standards as described in Table II, and the results reported as optical density.

Studies were also conducted to prove that the spots analyzed did, indeed, contain the amino acids, γ-aminobutyric acid, glutamic acid, glutamine, or alanine. Pooled brain extracts, before and after acid hydrolysis, were co-chromatographed with standards and with internal standards in the phenol-water solvent followed by the collidine-lutidine solvent or in the phenol-water system followed by 2-butanol-ethanol-ammonia. The spots of the unknowns and the standards run to the same position and the internal standards were recovered quantitatively. Further proof that the spot corresponding to glutamine on 2-butanol-ethanol-ammonia chromatograms contained only glutamine was obtained by chromatography of the eluate of this spot in the previously-mentioned 2-dimensional system. No ninhydrin-reacting constituent other than glutamine was found. Also, glutamic acid was the only ninhydrin-reacting substance found after acid hydrolysis of this eluate and subsequent chromatography in the same 2-dimensional systems.

Results. The effects of dieldrin and DDT on levels of gamma-aminobutyric acid, glutamine, glutamic acid, and alanine are illustrated in Tables I and II. As shown in the Tables, DDT had no significant effect on levels of gamma-aminobutyric acid, glutamic acid, alanine or glutamine. On the other hand, an increase of 24% was noted in the level of gamma-aminobutyric acid after administration of dieldrin. This increase was significant at level $p = 0.02$ to 0.01. This result is in agreement with recent studies(15, 18,23,33) that have shown that the occurrence of seizures is not necessarily related to decreased levels of gamma-aminobutyric acid in the whole brain. The present results do not rule out the possibility of localized decreases in the content of this amino acid that are not reflected in the concentration of gamma-aminobutyric acid in the whole brain.

However, the most striking change, easily visible on the chromatograms, was an aver-

TABLE I. Effects of Dieldrin or DDT on Concentration of Glutamine, Gamma-Aminobutyric Acid, and Glutamic Acid in Rat Brain. Nine control and 9 experimental animals were employed in studies with dieldrin; 8 control and 8 experimental animals in DDT experiments. Differences between control and experimental groups were tested for statistical significance by "Student's" T-test(37).

		Control group, mg/g brain			Experimental group, mg/g brain		
Toxicant		Glu NH$_2$	γ-ABA	Glu A	Glu NH$_2$	γ-ABA	Glu A
Dieldrin	Range	.33–.80	.14–.21	1.00–1.73	.32–1.00	.15–.26	.85–1.57
	Mean	.52	.17	1.32	.62	.21	1.22
	S.D.	.15	.027	.25	.21	.035	.25
	P				.3–.2	.02–.01	.5–.4
DDT	Range	.40–.56	.12–.19	1.00–1.53	.23–.79	.09–.21	.73–1.50
	Mean	.48	.16	1.23	.56	.16	1.15
	S.D.	.065	.022	.18	.19	.041	.32
	P				.3–.2	.8–.7	.6–.5

TABLE II. Effect of Dieldrin and DDT on Relative Alanine Content of Rat Brain.

		Dieldrin experiment, optical density at 570 mμ			DDT experiment, optical density at 570 mμ	
Chromatographic run		Controls	Dieldrin	Chromatographic run	Controls	DDT
1	Range	.105–.184	.190–.315	3	.070–.095	.070–.125
	Mean	.137	.248		.081	.090
	S.D.	.034	.053		.036	.024
	P		.02 –.01			.7 –.6
2	Range	.085–.115	1.50 –.225			
	Mean	.096	.189			
	S.D.	.013	.030			
	P		<.001			
1 and 2	P		.01 –.001			

Extracts of brains of 4 animals in control and dieldrin groups, respectively, were chosen at random and analyzed in each of the 2 chromatographic runs (Nos. 1 and 2) so that a total of 8 animals out of 9 in the respective groups of Table I were studied. Extracts of brains of 4 animals in control and DDT-poisoned groups, respectively, in Table I were selected at random and analyzed by 2-dimentional chromatography in Run No. 3. Differences between control and experimental groups in a given chromatographic run were tested for significance by "Student's" T-distribution as modified by Fisher(37) using logarithms of optical densities of the extracts. In the case of dieldrin in which 2 chromatographic runs were made, an additional test for statistical significance of the differences between experimental and control groups using the data obtained from both chromatographic runs was carried out by a modified "T" test.

$$t = \frac{E_1 - C_1 + E_2 - C_2}{\sqrt{\frac{ms^2}{n}}} \qquad S^2 = \frac{\Sigma(e_1 - E_1)^2 + \Sigma(c_1 - C_1)^2 + \Sigma(e_2 - E_2)^2 + \Sigma(c_2 - C_2)^2}{(n-1)m}$$

Where n = No. of determinations per mean; m = No. of means; e_1, and c_1 are the logarithms of optical densities on a given day of the samples in experimental and control groups, respectively. E_1 and C_1 are the means of the logarithms of optical densities of the samples in control and experimental groups on a given day. This equation was developed by Dr. Richard G. Cornell, CDC Statistics Section, and can be derived from the equation on page 85 of Snedecor(38).

age rise of 80% in concentration of alanine in the brains of rats given dieldrin. This was statistically significant at the level of $p = < 0.01$. The rise in concentration of this amino acid is probably the result of the inhibition by dieldrin of the oxidations of the Krebs tricarboxylic acid cycle in the brain, possibly at the α-ketoglutarate stage. The facts that DDT does not inhibit the respiration of mammalian brain(34,35) and does not change the level of alanine, glutamine, glutamic acid or γ-aminobutyric acid are both alpha

gamma

consistent with this hypothesis as to the action of dieldrin. However, a direct test of the effects of dieldrin upon the ability of rat brain to oxidize members of the Krebs cycle other than succinate(36) has not been carried out.

Summary. Studies were made of the effects of lethal doses of dieldrin or DDT on the free amino acid pattern of the brains of rats exhibiting severe symptoms of poisoning. Elevated levels of α-alanine and γ-aminobutyrate were noted with dieldrin but not with DDT, indicating a different mode of action of the 2 insecticides.

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Screening of Various Adsorbents for Protection against Paraquat Poisoning

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by
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The herbicide paraquat has been used extensively in the United States for weed control for several years. It is available to farmers primarily as paraquat dichloride (1,1'-dimethyl-4,4'-bipyridinium dichloride) 29.1% liquid concentrate containing 2 lbs paraquat cation per gallon. However, 42% liquid concentrate paraquat bis(methylsulfate)-(1,1'-dimethyl-4,4'-bipyridinium bis(methylsulfate) has been used to some extent. Since paraquat was first introduced into agricultural practice a few ill-effects in applicators have occurred in this country, mostly due to local irritant effects of the caustic material on the skin or in the eyes. One reported nonfatal case of ingestion presented signs of superficial ulceration of the oropharynx (PASI and HINE 1971). In the United Kingdom where the herbicide has been used for a much longer period of time, a number of serious poisonings and deaths have been reported as a result of having accidentally or intentionally ingested relatively small amounts of the liquid concentrate formulation (BULLIVANT 1966; CAMPBELL 1969; CARSON 1969; CLARK et al. 1966; DUFFY 1968; FENNELLY et al. 1968; KERR et al. 1968; MATTHEW et al. 1968; MOURIN 1967; OREOPOLULES et al. 1968). The deaths occurred primarily as a result of irreversible pathological changes in the lungs.

The main therapeutic efforts in ingestion cases are directed at reducing absorption of toxin from the GI tract via lavage, cathartics and adsorbents. Much of the research on the removal, by adsorption, of accidentally ingested drugs and poisons has involved the use of activated charcoal (ANDERSON 1948; CHIN et al. 1969; DECKER et al. 1968, 1968a, 1969). Some recent work, however, has pointed to the value of investigating substances other than charcoal for removal of accidentally ingested toxins such as paraquat (BROWNE 1971; CLARK 1971). The purpose of the present study was to screen various materials to assess their potential as adsorbents for ingested paraquat in an effort to determine the most suitable materials for further study on experimental animals or human beings. Since the completion of this study, CLARK (1971) reported Bentonite and Fuller's Earth to be effective adsorbents in vivo as well as in vitro. Results of the present study are submitted for any who may wish to further explore adsorption of paraquat by various materials.

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EXPERIMENTAL

Screening of adsorbents was carried out in three phases. The first phase involved initial screening of 25 different materials to determine which were most suitable for further study. The procedure used was as follows: One-half ml of paraquat liquid concentrate (29.1% paraquat cation) was added to 20 ml of simulated gastric juice made up according to the formula of GUDIKSEN (1943). This was followed by the addition of 10 ml of an aqueous slurry containing 5 gm of adsorbent. The flask was then shaken for 20 minutes using a Burrell wrist action shaker while being maintained at a temperature of 37°C in a constant temperature bath. After shaking, the solution was separated from the solid contents of the flask either by filtration through Whatman No. 42 filter paper or by centrifugation. One ml of this clear solution was diluted to 100 ml with water. Finally, 2 ml of 0.2% sodium dithionite in 0.2 N NaOH solution was added to 10 ml of this dilution. The developed color was read immediately at 395 mμ in a spectrophotometer. Optical density readings were converted to percent paraquat removed from the simulated gastric solution.

Information obtained from initial screening in the first phase was used to determine the adsorptive materials to be tried in the second phase. In order to simulate experimental conditions which might result during accidental ingestion of paraquat into the stomach and supportive first-aid treatment, the procedure described by DECKER et al. (1968) regarding removal of various drugs by activated charcoal was followed. In this procedure 100 ml of artificial gastric juice was placed in a 250 ml Erlenmeyer flask incubated at 37°C. Two ml of the paraquat concentrate (29.1% paraquat dichloride) were added directly to the flask and the contents shaken for 1 minute with a Burrell Wrist action shaker. A slurry of 5 gm of the adsorbent in 50 ml of water was then quantitatively transferred to the flask and incubation with shaking was continued. After 20 minutes (a simulated elapsed time from accidental ingestion until definitive treatment such as gastric lavage accompanied by supportive therapy could be administered) the adsorbent was separated from the contents of the flask either by filtration through Whatman No. 42 filter paper or by centrifugation. Analysis of the resultant solution was accomplished by first taking 1 ml of the filtrate and diluting to either 100 ml or 1000 ml (depending on the activity of the adsorbent for removing paraquat). Next, to 10 ml of this diluted solution was added 2 ml of 0.2% sodium dithionite color developing solution. The developed blue color was read immediately in a spectrophotometer at 395 mμ. The higher the percent paraquat removed from solution the greater the protection given by the adsorbent.

The third phase of this experiment was conducted using Amberlite resin which was shown to be the most effective adsorbent in the second phase of the experiment. The method used to obtain the data was the same as that used in the second phase of screening.

RESULTS AND DISCUSSION

The paper presents

Table 1 summarizes the results of initial screening for 25 different adsorbents and indicates which were most suitable for further study. The list includes a number of readily available pharmaceutical or household materials as well as a few adsorbent materials commonly found in chemistry laboratories. Eight of the more promising adsorbent materials, plus one additional material not used in the initial screening, were compared in the second phase of the experiment. As can be seen in Table 2, Amberlite CG-120 resin showed much greater adsorptive capacity for paraquat in simulated gastric juice than any of the other materials. Removal of paraquat from the experimental gastric juice was over 99% with this adsorbent as compared with 59.7% for an equal weight of the material next in efficiency.

A more thorough evaluation of the activity of the Amberlite resin was carried out in the third phase of the experiment. Results are shown in Table 3. Amberlite CG-120, 200-400 mesh, was used because of its slightly better suspending properties than the 100-200 mesh type. It has about equal effectiveness with the 100-200 mesh type as an adsorbent. The volumes of 2 to 30 ml of paraquat concentrate used in this part of the experiment were amounts considered to be in the range of what a human being might accidentally ingest. The volume of 5 ml is commonly accepted for a teaspoonful and 15 ml for a tablespoonful. The amount of Amberlite resin required to effectively neutralize the paraquat was varied in order to find the greatest percent removed with the least amount of adsorbent.

The results of this study indicate that, of the materials tried, Amberlite CG-120, either 100-200 or 200-400 mesh, is the most promising adsorbent for study on experimental animals. A limited amount of exploratory animal work at this laboratory has shown the Amberlite resins to be effective in reducing tissue levels compared with nonprotected animals and, in some cases, there was indication of protection against poisoning.

Use of trade names is for identification purposes only and does not constitute endorsement by the Environmental Protection Agency.

TABLE 1

Paraquat Adsorption Screening Test

Adsorbent	Final Concentration[a] of Paraquat in Gastric Solution ($\mu g/ml$) x 10^3	Percent Paraquat Removed from Gastric Solution
Amberlite CG-120 (ion exchange resin, 100-200 mesh)	0.03	99.4
Res-Q, universal antidote[b]	0.03	99.4
Amberlite CG-120 (ion exchange resin, 200-400 mesh)	0.05	99.1
Magnesium Trisilicate, U.S.P. powder	0.05	99.1
Charcoal, Norit A	0.07	98.6
Charcoal, Coconut (activated, 50-200 mesh)	1.05	81.2
Florisil, P.R. grade	1.34	74.6
Nuchar Attaclay	2.50	53.2
Gelusil, tablets[c]	2.74	48.3
Silica Gel G (grain size 10-40μ)	4.79	9.7
Alumina, activated (Alcoa Type F-1, 60-80 mesh)	4.79	9.7
Kaopectate, liquid	4.92	7.2
Soil, sandy loam	4.92	7.2
Aluminum Oxide G (grain size 10-40μ)	4.99	5.8
Alumina, absorption (80-200 mesh)	5.09	4.0
Charcoal, animal, powder	5.16	2.6
Jello	5.16	2.6
Soil, potting (high humus content)	5.16	2.6
Pectin	5.18	2.3
Chromosorb W, DMCS-A/W (60-80 mesh)	5.19	2.0
Milk of Magnesia, tablets	5.19	2.0
Silicic Acid (325 mesh)	5.19	2.0
Sand, mortar	5.28	0.3
Milk of Magnesia, liquid	5.30	0.0

[a] Values were determined by taking a 1/100 dilution of the original paraquat and gastric solution, developing the color with sodium dithionite and reading the optical density at 395 mμ. Calculations were based on a standard recovery curve. Determinations were run in duplicate. Initial concentration of paraquat in the simulated gastric juice was 5.3 x 10^3 $\mu g/ml$.

[b] A universal antidote for accidental poisoning containing activated charcoal, magnesium hydroxide, and tannic acid.

[c] A pharmaceutical preparation containing a mixture of magnesium trisilicate and aluminum hydroxide, ratio 2:1.

TABLE 2

Paraquat Gastric Adsorption by Selected Materials

Adsorbent	Final Concentration of Paraquat in Gastric Solution (μg/ml) 1 x 1000 dilution[a]	Percent Paraquat Removed from Solution[b]
Amberlite CG-120 (100-200 mesh)	20^{c}	99.3
Amberlite CG-120 (200-400 mesh)	25^{c}	99.1
Res-Q (universal antidote)	1.25×10^3	59.7
Charcoal, Coconut (50-200 mesh)	1.6×10^3	48.4
Magnesium Trisilicate (U.S.P. powder)	1.75×10^3	43.6
Charcoal, Norit A	1.95×10^3	37.1
Gelusil, tablets (finely powdered)	2.2×10^3	29.1
Florisil, P.R. grade	2.55×10^3	17.7
Kaolin (hydrated aluminum silicate, N.F. colloidal)	3.1×10^3	0.0

[a] These values were determined by taking a 1/1000 dilution of the original paraquat and gastric juice mixture, developing the color with sodium dithionite and reading the optical density at 395 mμ. Calculations were made from a standard recovery curve.

[b] Average of three values. Initial concentration of paraquat in the simulated gastric juice was 3.1×10^3 μg/ml.

[c] For both the Amberlite resin adsorbents only a 1/100 dilution was needed to obtain a measurable reading in the spectrophotometer.

TABLE 3

Paraquat Gastric Adsorption Using Amberlite

Volume of Paraquat Concentrate (ml)	Amount of Amberlite Adsorbent (gm)	Initial Amount of Paraquat Cation (gm)	Final Amount of Paraquat (gm/ml of dilution)[a]	Percent Removed[b]
2	5	.471	.0065[c]	98.6
3	5	.707	.014[c]	98.1
4	5	.942	.045[c]	95.2
5	5	1.18	.115	90.3
6	5	1.41	.253	82.1
8	5	1.88	.566	70.0
9	10	1.88	.138	92.7
10	5	2.36	1.14	51.4
10	10	2.36	.26	88.8
10	15	2.36	.088	92.3
10	20	2.36	.038	98.4
15	10	3.53	.007	66.5
15	15	3.53	.003	86.4
15	20	3.53	.0014	93.4
30	20	7.05	.013[d]	64.4
30	30	7.05	.007	80.8
30	40	7.05	.0038	89.4

[a] Dilution 1/1000 except where noted otherwise.
[b] Average of three values.
[c] Required a dilution of 1/100.
[d] Required a dilution of 1/10,000.

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Absorption and Excretion of Parathion by Spraymen

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Significant amounts of the urinary metabolite *p*-nitrophenol were detected in the urine of spraymen as long as ten days after last exposure to the organophosphorus insecticide, parathion. Approximately two days after exposure, excretion was insignificant during late night and early morning hours, but reached higher levels during midday. The height of immediate postexposure excretory peaks and the delayed midday rises in excretion seemed to vary directly with the temperature. Bathing after exposure was associated with a rapid decrease in *p*-nitrophenol excretion. Tests considering only one route of exposure at a time indicated that the dermal route represents a potentially greater source of absorption than the respiratory route for orchard spraymen using liquid parathion formulations under the conditions of this study. However, with equivalent absorbed dosages the respiratory route is the more hazardous.

THE organic phosphorus insecticide parathion is a compound of known high acute toxicity to experimental animals. Its use during the more than 20 years since its introduction has resulted, for persons occupationally exposed, in accidental poisonings, some of which have been fatal. The development of effective safety recommendations for workers using parathion and other highly toxic pesticides requires information as to the relative importance of the various possible routes of exposure which might be involved. In those cases of accidental occupational poisoning which occur, it is usually impossible to determine if the exposure was predominantly respiratory or predominantly dermal. However, there is some experimental evidence to indicate that, at least under certain conditions, potential exposure of spraymen to parathion is predominantly by the dermal route. Using the dermal and respiratory exposure pad and air sampling techniques, Batchelor and Walker[1] found that dermal contamination was considerably greater than respiratory contamination for orchard spraymen. Similar results which indicate a preponderance of dermal over respiratory contamination have been obtained for mosquito control spraymen applying malathion or *O*-(3-chloro-4-nitrophenyl) *O*, *O*-dimethyl phosphorothioate (Chlorthion) aerosol[2]; entomologists checking azinphosmethyl or methyl-parathion-treated cotton[3]; orchardists applying sodium salt of dinitro-*o*-cresol (Na-DNOC) as an apple thinning spray[4,5] or DDT[6]; workmen applying the herbicide 2,4-dinitro-*o*-*sec*-butylphenol (DNOSBP)[5]; operators formulating or spraying azinphosmethyl,[7-9] endrin, malathion, parathion, or carbaryl with ground equipment

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or endrin by air[9]; workmen applying lindane or DDT to forest land[10]; and vector control spraymen applying dieldrin[11] or DDT.[12]

On the other hand, studies of volunteers have revealed a lack of toxic effect from large dermal doses of parathion,[13] although parathion applied to the skin of laboratory animals has shown high toxicity,[14] and one human case of severe parathion poisoning has resulted from dermal application of the compound in an attempt to relieve pruritus.[15] In the studies on volunteers, the absorption of large amounts of parathion or its breakdown products following dermal dosing was confirmed by the finding of high levels of *p*-nitrophenol in the urine (up to 5.8 mg/24-hr sample), but little or no associated cholinesterase depletion and no cholinergic signs or symptoms occurred. However, the same investigators were able, under other conditions of dosage, particularly by the respiratory route or by a combined respiratory-dermal administered dosage, to produce significant cholinesterase depletion as well as serious poisoning in volunteers.[13,16]

Hartwell and Hayes[16] showed that, if to formulating plant workers or crop-dusting pilots exposed to parathion and mevinphos respiratory protection was provided in the form of pure compressed air for breathing, the measure was effective in preventing poisoning as well as in reducing noncritical exposure as measured by blood cholinesterase activity level.

The oral route of exposure has generally been considered to be of minor significance for workmen using toxic pesticides. However, Quinby et al[17] have described the serious poisoning of a parathion sprayman whose exposure was thought to have occurred from eating a candy bar which he had carried all day in an open pocket during his spray activities. These authors presented comparative curves from poisoning cases which showed that *p*-nitrophenol excretion decreased much more rapidly after oral exposures than after dermal exposures.

The present study, using urinary excretion of *p*-nitrophenol as an indicator, was carried out to obtain additional information on the exposure of spraymen to parathion and particularly, to obtain more information on the relative importance of dermal vs respiratory routes of exposure.

Materials and Methods

Excretion of *p*-Nitrophenol by Spraymen.—Spraymen studied for *p*-nitrophenol excretion included 16 orchardists from the Wenatchee Valley of the state of Washington. These men were operators of power airblast spray equipment, applying 0.33% liquid parathion (1 lb of 25% water-wettable powder per 100 gal of water) formulation to fruit orchards. The study involved 33 spraying days and further observations for an additional 64 days. The men kept a complete record of their activities while participating in the study. The record included the time spent spraying parathion, other work which might involve exposure to parathion, and the bathing schedules of the men. They made complete 24-hour collections of urine for several days during and following their exposure to parathion. Each voiding was kept in a separate container, and the time of excretion was recorded. A total of 514 urine samples was analyzed for *p*-nitrophenol. Samples were analyzed for *p*-nitrophenol content by the method of Elliott et al.[18] Results were calculated as *p*-nitrophenol excretion per hour.

Potential dermal exposure of these spraymen to parathion was estimated using the absorbent pad technique described by Durham and Wolfe.[6] Potential respiratory exposure for the time the worker actually sprayed was calculated by using the mean parathion respiratory exposure of 0.020 mg/hr, which had been determined in earlier studies for spraymen operating airblast equipment.[19] During the actual period of study, respiratory measurement could not be made by this method without severely reducing the respiratory absorption of parathion by the subject. The technique for estimating potential dermal absorption involved placement of pads on clothed body parts which were in close approximation to the bare skin to be studied. Thus, the pad did not significantly influence the total dose received by means of the dermal route.

Comparative Dermal and Respiratory Exposure to Parathion.—In special controlled studies of the comparative dermal and respiratory exposure of workers subjected to the mist emitted from an airblast spray machine during parathion application in orchards, the technique developed at the Phoenix Field Station, National Communicable Disease Center, Public Health Service,[13] was used. In this procedure, one individual was completely covered with rubber and plastic clothing to prevent skin contamination. He breathed ambient air and, thus, had respiratory exposure only. A second worker breathed pure compressed air but wore ordinary clothing, so that he had dermal exposure only. The third person had neither respiratory nor dermal protection, other than ordinary clothing. The workers wore absorbent pads to provide another measure of their potential, or real, dermal exposure. The three individuals were about equally exposed to a parathion spray drift by transport in a jeep following the spray machine at a distance such that the spray mist was fine enough to remain suspended in the air for a comparatively long period. Exposure to this fine mist, rather than the course droplets encountered near the rear of the spray machine, was similar to that encountered by the operator of such equipment. Urine samples were collected for 24 hours by the three subjects, and the *p*-nitrophenol excretions were determined and compared. Two replicates of this test were carried out.

The Smyth technique, described in detail by Durham and Wolfe,[20] was used to obtain additional values for comparing dermal and respiratory absorption for field spraymen. It involves estimation of total absorption of a chemical from measurement of the chemical or its biotransformation products in the excreta. Respiratory absorption was assumed to be equivalent to respiratory exposure. Respiratory exposure for five subjects was based on mean value for special respirator pads from spraymen exposed under comparable conditions. Respiratory exposure for the sixth subject was based on air concentration of parathion and an assumed tidal volume and respiratory rate. Dermal absorption was calculated by difference between total absorption and estimated respiratory absorption.

Results

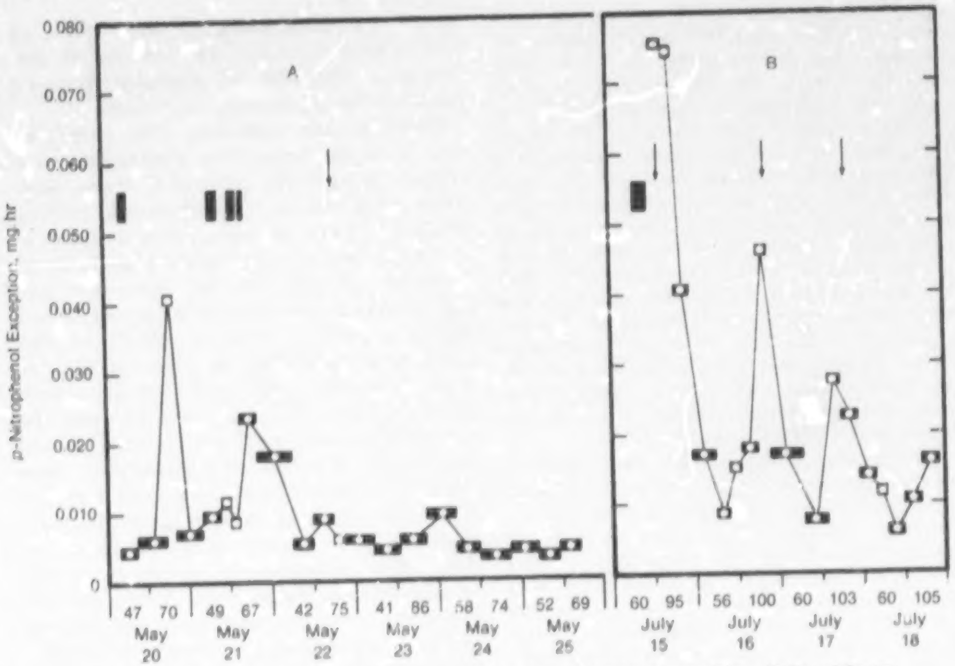
Excretion of *p*-Nitrophenol by Spraymen Exposed to Parathion Under Field Conditions.—The Figure illustrates two examples, from the total of 16 spraymen studied, of excretion of *p*-nitrophenol by two workers exposed to parathion. From a study of these curves, and others not illustrated, a number of conclusions were drawn regarding the relationship between exposure to parathion and excretion of *p*-nitrophenol.

In almost every instance in which a sprayman was studied, there was a significant increase in the excretion of *p*-nitrophenol in association with the exposure period. The highest excretion occurred, on the average, about six hours after the last exposure. However, in some instances, particularly in cases where spraying was prolonged, the highest excretion occurred soon after or even before, exposure was terminated. Excretion of *p*-nitrophenol generally decreased rapidly after the postexposure peak.

Bathing after exposure was associated with a rapid decrease in *p*-nitrophenol excretion. Apparently, removal of excess parathion from the skin by thorough bathing limited further absorption of the insecticide. Although bathing may cause a decrease in *p*-nitrophenol excretion, the spraymen usually bathed in the evening when one would expect a decrease to occur. Of 51 evening baths recorded during the study, all but one were associated with decreases in *p*-nitrophenol output. Decreases also followed the six daytime baths recorded. In many cases, appreciable amounts of *p*-nitrophenol were detected in the urine as long as four or five days after exposure, depending on the magnitude of the exposure. However, about two days after exposure, the excretion of this metabolite was insignificant during the late night and early morning hours, but reached higher levels during the middle of the day. The higher temperature, the greater activity at midday, or both, may have contributed to the more rapid absorption and consequent excretion of parathion during this period. After his period of spraying was completed, each person who was under study was urged to refrain from wearing contaminated clothing and to minimize other exposure to a contaminated environment. In most instances it was impossible to isolate the subject completely from all environmental exposure to parathion, since he usually had to enter the orchard for brief periods to change irrigation sprinklers or to do other tasks. However, in the few instances in which the subject was completely out of all environmental contact with sprays, the diurnal excretion pattern persisted.

The height of immediate postexposure excretory peaks and of the delayed midday rises in excretion also seemed to vary directly with temperature. This variation is most apparent when one compares the hot days of July with the cooler days of May. This observation strengthens the possibility that the diurnal variation noted in the *p*-nitrophenol excretory pattern may be related to diurnal changes in temperature.

None of the *p*-nitrophenol excretion levels



Excretion of p-nitrophenol by two spraymen (A and B) exposed to parathion. Daily minimum and maximum temperatures, in degrees Fahrenheit, shown for each date. Sample period represents interval covered by collection of a voiding of urine (total number of hours per voided specimen). Spray period (solid blocks); bath (arrows); sample period (open circle blocks) as indicated.

of the spraymen studied approached the high values sometimes seen in asymptomatic workers, much less the levels seen in poisoned adults. The highest rate of excretion of *p*-nitrophenol noted in this study was 0.151 mg/hr (2.4 ppm). This value may be compared with the peak level of 3.0 ppm and the mean level of 1.6 ppm for spraymen (part-time ground applicators) reported by Arterberry et al.[21] In general, the spraymen who were included in the present study were careful workers, selected as persons who would keep the accurate records and complete urine samples required. On the whole, they probably paid more attention to safety precautions than the average sprayman.

Comparative Dermal and Respiratory Exposure to Parathion.—As can be seen in Table 1, results were similar in each of the two tests involving measurement of comparative dermal and respiratory exposure of workers under controlled conditions.

In test 1, analysis of dermal exposure pads from right shoulder, left shoulder, back, and chest areas confirmed that all three workers were subjected to spray mist contamination of about the same order of magnitude. The average parathion concentration on the pads was 0.031 mg/sq in for the man wearing the plastic suit, and 0.029 mg/sq in for the man using the pure air supply. The man wearing no protection was subjected to less potential dermal exposure (0.017 mg/sq in) than the other two subjects.

The total *p*-nitrophenol excretion for the man wearing the plastic suit (respiratory exposure only) was 0.088 mg; for the individual using the pure air supply (dermal exposure only), 0.666 mg; and for the man using no special protective equipment (both respiratory and dermal exposure), 0.433 mg.

In test 2, analysis of exposure pads confirmed that total potential spray impingement was about equal for the man wearing the plastic suit (0.020 mg/sq in) and for the man provided with the pure air supply (0.019 mg/sq in). However, the total *p*-nitro-

Table 1.—Potential Skin Contamination and Total p-Nitrophenol Excretion While Wearing Various Protective Equipment Items*

Subject	Protective Equipment	Route of Exposure	Potential Skin Contamination, Parathion, mg/sq in Test 1	Potential Skin Contamination, Parathion, mg/sq in Test 2	Total p-Nitrophenol Excretion, mg Test 1	Total p-Nitrophenol Excretion, mg Test 2
1	Plastic and rubber clothing	Respiratory only	0.031†	0.020†	0.088	0.006
2	Pure air supply	Dermal only	0.029	0.019	0.666	0.497
3	0	Respiratory and dermal	0.017	...	0.433	...

* For workers exposed to parathion spray mist.

† Since this subject's skin was covered by plastic and rubber clothing, he could not absorb any of this potential dosage. Quantity was measured and illustrates similarity in exposure conditions for various subjects.

Table 2.—Calculated Dermal Absorption of Parathion by Workers Spraying Orchards

Subject	Total Urinary Parathion Excretion, mg	Respiratory Exposure mg/hr	Respiratory Exposure hr	Respiratory Exposure Total, mg	Dermal Exposure mg/hr	Dermal Exposure hr	Dermal Exposure Total, mg	Calculated Dermal Absorption (Excretion Minus Respiratory Exposure) Total, mg	Proportion of Total Excretion, %	Proportion of Total Dermal Exposure, %
1	0.67	.020*	6.25	0.13	13.6	6.25	85.0	0.54	80.6	0.63
2	1.50	.020*	7.25	0.15	14.1	7.25	102.2	1.36	90.7	1.33
3	1.49	.020*	7.50	0.15	12.6	7.50	94.5	1.34	89.9	1.41
4	1.36	.020*	7.75	0.16	9.5	7.75	73.6	1.20	88.2	1.63
5	0.51	.020*	5.00	0.10	20.4	5.00	102.0	0.41	80.4	0.40
6	7.91	.055	13.75	0.76	26.9	13.75	369.9	7.15	90.2	1.95
Mean	...	...	...	0.24	...	...	137.9	2.00	86.7†	1.23†

* Mean respiratory exposure for airblast spray equipment operators.

† Based on individual proportions.

enol excretion was 0.006 mg for the for- er (respiratory exposure only) and 0.497 g for the latter (dermal exposure only). A bject with no protective equipment was t included in this test.

The results of these tests indicate that to chard spraymen using liquid parathion mulations, under the conditions of this dy, the dermal route may represent a eater source of absorption than the re- iratory route. This should not, however, construed to mean that workers should glect respiratory protection where there is piratory exposure because at any given sage rate respiratory exposure is more ngerous, inasmuch as by this route the ticide is more readily and more complete- absorbed.

he data obtained by the application of Smyth technique[20] to exposure and ex- tion studies in field spraymen are presented in Table 2. In each of the spray exposures in this study the calculated dermal absorption was greater than the estimated respiratory absorption. The calculated dermal absorption represented, on the average, 87% of the total absorption. However, the calculated dermal absorption represented only a small percentage (range, 0.40% to 1.95%; mean, 1.23%) of the measured potential dermal exposure.

Comment

The use of *p*-nitrophenol excretion as a measure of absorption of parathion seems to be a valid procedure. The applicability of this measurement to dermal absorption of parathion has been strengthened by the work of Fredriksson et al,[22] who reported that parathion was not hydrolyzed or transformed into paraoxon by the skin of man, cat, rabbit, or rat and by Nabb et al,[23] who found evidence that parathion, when applied dermally to rabbits, reaches the blood stream largely unaltered. Arterberry and his colleagues[21] have shown that there is a reasonably good correlation between *p*-nitrophenol excretion and exposure to parathion, change in blood cholinesterase activity level, and the occurrence of poisoning. The greater sensitivity of *p*-nitrophenol excretion as compared to blood cholinesterase activity level for measuring exposure to parathion has made possible studies of parathion metabolism at quite low levels of exposure. A particularly sensitive method for detection of *p*-nitrophenol has been developed by Cranmer.[24]

Baetjer and Smith[25] have reported that parathion is more toxic to mice at higher environmental temperatures. The temperature effect was less marked when the parathion was injected intravenously than when it was given intraperitoneally. The authors interpreted this difference as an indication that variation in the rate of absorption was probably the most important factor causing the increased toxicity at higher temperature.

Physicians and others in certain agricultural areas have observed for a long time that poisoning from the organic phosphorous insecticides occurs more often in unusually hot weather than under cooler conditions. However, more spraying is done in the warmer weather; therefore, any valid conclusion, based on epidemiologic data relating to the effect of temperature on poisoning, would have to take into account the intensity of exposure and the size of the population at risk. Our present studies with exposed workers also indicate that, after dermal exposure to parathion, greater excretion of *p*-nitrophenol is associated with higher temperature. This effect was shown not only in connection with diurnal variations but also in connection with seasonal variations in temperature. The seasonal difference in excretion of *p*-nitrophenol tends to discount the importance of muscular work and other activity which might be considered as a factor in the observed diurnal difference in excretion of *p*-nitrophenol. Furthermore, the relationship observed by Funckes et al[26] and by Baetjer and Smith[25] depended on temperature, not on activity.

Diurnal variation in excretion of *p*-nitrophenol has been observed only after dermal exposure to parathion, probably because absorption is relatively prolonged after dermal exposure, as compared with oral exposure.[17]

The studies of dermal absorption of parathion reported above indicate that absorption by this route is both slow and incomplete and that the rate of this absorption is probably increased by higher temperatures. There may be other factors which, under certain circumstances, also may act to increase the rate of dermal absorption. It is generally recognized that injury to the skin may greatly increase its permeability.[27] In addition, there are conditions that may modify the reaction of the host organism to the absorbed toxicant. One of the most commonly recognized conditions is previous exposure to an anticholinesterase compound.

It is of interest to note Fredriksson an Bigelow's study[28] of the tissue distributio of parathion labeled with phosphorus 32 i the mouse, following subcutaneous injection These workers stated that radioactivit (representing stored parathion) was low, o absent, in the skin among other tissue However, study of their original slides indicates that appreciable levels of parathio were present in the skin.

The evidence presented here indicat that, under the conditions of this study orchard spraymen using parathion in a airblast type of spray machine, skin contamination is potentially a more importan route of absorption, but not necessarily poisoning, than the respiratory route. Thi conclusion may not be valid for other compounds, or even for parathion under different conditions of use. However, the presen work does extend the original observation Batchelor and Walker[1] one additional ste Their work showed that much larg amounts of parathion impinged on the ski than were breathed into the lungs. The results presented here confirm those of Durham and Wolfe[29] and indicate that, eve when the more rapid and more nearly complete absorption by the respiratory route considered, the skin still represents an important route of entry for parathion or i metabolite.

On the basis of these results, precautio for use of parathion should emphasize t importance of protecting the skin from exp ure. Spraymen should also be encouraged to use respirators to prevent respiratory absorption.

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THE EFFECT OF POLYCHLORINATED BIPHENYLS ON RAT REPRODUCTION

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Abstract—Reproduction, pathology and acute toxicity were studied in Sherman rats exposed to the polychlorinated biphenyls, Aroclor 1254 and Aroclor 1260. Rats exposed to Aroclor 1254 at dietary levels of 20 ppm or more had fewer pups per litter than the controls in the F_{1b} and F_2 generations. The 100 ppm exposure level of Aroclor 1254 increased mortality in the F_{1b} offspring and markedly decreased mating performance of the F_{1b} adults. The 500 ppm dietary level of Aroclor 1260 reduced litter size and decreased survival in the F_1 litters. Dietary levels of 5 ppm Aroclor 1254 and 100 ppm Aroclor 1260 had no effect on reproduction in rats exposed through two generations. Liver weights were increased in 21-day-old F_1 male weanlings at the 1 ppm level of Aroclor 1254 and in either sex of F_1 and F_2 weanlings at 5 ppm or higher levels of both Aroclor 1254 and 1260. Histological changes in the liver and increased liver weights were observed in adult rats exposed to the higher levels. Pregnant rats given Aroclor 1254 at the rate of 100 mg/kg/day on days 7–15 of gestation produced grossly normal litters, but only 30·1% of the pups survived to weaning. Reproduction and pup survival were not affected at dosage rates of 50 mg. Aroclor 1254/kg/day or 100 mg Aroclor 1260/kg/day. Oral LD_{50} values in 3–4-wk-old male rats were 1295 and 1315 mg/kg for Aroclors 1254 and 1260, respectively. The iv LD_{50} for Aroclor 1254 in adult females was 358 mg/kg.

INTRODUCTION

Reproductive defects due to commercial mixtures of polychlorinated biphenyls (PCBs) have been reported in experimental birds (Dahlgren, Linder & Carlson, 1972) and mammals (Ringer, Aulerich & Zabik, 1972). Porphyria (Vos & Koeman, 1970), microsomal-enzyme induction (Street, Urry, Wagstaff & Blau, 1969) and liver damage (Kimbrough, Linder & Gaines, 1972; Miller, 1944) have also been reported in experimental animals exposed to PCB mixtures.

The present investigation was initiated in 1970 to study the effects on reproduction and pathology produced by two American-made PCB mixtures sold under the trade-names Aroclor 1254® and Aroclor 1260®. Aroclor 1254 contains 54% (w/w) chlorine and is composed of 11% tetra-, 49% penta-, 34% hexa- and 6% heptachlorobiphenyls; Aroclor 1260 has 60% (w/w) chlorine, with a composition of 12% penta-, 38% hexa-, 41% septa-, 8% octa- and 1% nonochlorobiphenyls (Thruston, 1971). An almost complete identification of isomers in Aroclor 1254 and Aroclor 1260 has been obtained (Sissons & Welti, 1971). Since 1970, the Monsanto Co., the only US manufacturer of PCBs, has voluntarily reduced sales of Aroclor mixtures and has taken steps to limit their use to closed systems (Monsanto Co., 1971).

The present communication is an account of reproduction studies in rats. Also included are acute toxicity values from preliminary studies and comments on pathology and haematology in animals from the reproduction experiments. A detailed account of the liver pathology of the F_0 rats given 20 ppm and higher dietary levels has been published (Kimbrough *et al.* 1972).

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EXPERIMENTAL

Animals and materials. All animals used in the studies were Sherman-strain rats produced under specific-pathogen-free conditions. Aroclor 1254 (Lot AK-38) and Aroclor 1260 (Lot AK-3) were supplied by Monsanto Industrial Chemicals Company, St. Louis, Mo.

Experimental design and conduct

LD$_{50}$ studies. The single-dose oral LD$_{50}$ was determined for Aroclor 1254 and 1260 in weanling male rats (3–4 wk old). The iv LD$_{50}$ was determined in adult females for Aroclor 1254. Between five and eight groups, each of ten rats, were used to obtain each LD$_{50}$ value. The procedure described by Gaines (1969) was followed for oral dosing; Aroclor 1254 or Aroclor 1260 was dissolved in peanut oil and the solution was administered by oral intubation at the rate of 5 ml/kg. For iv dosing, Aroclor 1254 was first dissolved in peanut oil. One part of this solution was added to nine parts of a 1% lecithin–saline suspension and homogenized. This formulation was administered in a single injection via the tail vein at the rate of 5 ml/kg. A control group of five rats received 10% peanut oil in lecithin–saline in the same volume. Survivors were observed for 10–14 days.

Two-generation reproduction studies. For these studies the rats were fed either untreated ground laboratory chow (control) or ground chow fortified with Aroclor 1254 or Aroclor 1260. The Aroclors were dissolved in ether and mixed with cornstarch. The ether was allowed to evaporate and the Aroclor–cornstarch mixture was mixed into increasing amounts of ground chow. Aroclor 1254 was fed at dietary levels of 0 (control), 1, 5, 20 and 100 ppm, while Aroclor 1260 was fed at levels of 0, 5, 20 and 100 ppm. Because exposure schedules had to be staggered, a specific control group was added each time one or more treated groups were started on a dietary regimen. The F$_0$ rats were started on the diets at 3–4 wk of age and the F$_{1b}$ rats at weaning. Exposure was continuous through mating, gestation and lactation until the rats were killed. Ten males and 20 females were fed at each dietary level in both the F$_0$ and F$_{1b}$ generations. The F$_0$ males at each dietary level and half of the females were caged individually. Other F$_0$ females and all the F$_{1b}$ rats were caged in groups except during the reproduction cycle. Body weights were recorded weekly on F$_0$ rats, except during the reproduction phase, and food consumption was measured during wk 2 and 5, the week before each mating and the week after weaning of the F$_{1a}$ litters. F$_{1b}$ rats were weighed when started on the diet, during the week before mating and at sacrifice.

The F$_0$ rats were pair-mated when 3 and 7 months old to produce the F$_{1a}$ and F$_{1b}$ generations, respectively. Breeding-stock F$_{1b}$ rats were selected at weaning from all available litters and pair-mated when 3 months old to produce the F$_{2a}$ generation. F$_{1b}$ rats on dietary levels of 0, 20 and 100 ppm Aroclor 1254 were mated a second time when 8 months old to produce the F$_{2b}$ generation. Viability counts of offspring were made at birth, day 3, day 7 and day 21 (weaning). Litters were inspected daily for condition and the presence of dead pups. After the F$_{1b}$ offspring had been weaned, ten adult F$_0$ rats of each sex were killed and their livers were weighed and fixed for histological examination. Following the weaning of the F$_{2b}$ generation, for Aroclor 1254, and the F$_{2a}$ generation, for Aroclor 1260, haematological values (total leucocyte count, haematocrit, haemoglobin and differential leucocyte count) were determined on ten adult F$_{1b}$ rats of each sex at the dietary levels of 0, 20 and 100 ppm. These rats were then killed and weights of the spleen, heart, lungs, brain, kidneys, testes and liver were recorded. Tissues were fixed for microscopic study.

At lower dietary levels, haematology was not done and only the livers were weighed. At dietary levels of 5 ppm or less of both Aroclors, one male and one female weanling from each of ten litters from each generation were killed at 21 days of age and the livers were weighed. F_{2a} weanlings at dietary levels of 0, 20 and 100 ppm Aroclor 1254, and F_{1b} and F_{2a} weanlings at levels of 0, 20 and 100 ppm Aroclor 1260 were killed in the same manner and the livers were fixed for further study.

One-generation reproduction studies. Preliminary studies were conducted on both Aroclors at dietary levels of 0, 100 and 500 ppm. The experimental design was the same as that described for the two-generation studies, except that only ten females were used. This study was terminated after the F_{1b} offspring had been weaned. Rats fed 500 ppm Aroclor 1254 were bred only once. Haematology values and organ weights were determined and tissues were examined microscopically on the F_0 rats.

Post-implantation exposure studies. Stock rats 100 days old were pair-mated. Insemination was verified by microscopic inspection of a daily vaginal smear. Counting the day of insemination as day 0, the females were dosed by oral intubation on days 7–15 of gestation (a total of nine doses). To maintain a constant dose volume on a body-weight basis, Aroclor 1254 or 1260 was dissolved in peanut oil at appropriate concentrations and these formulations were administered at the rate of 5 ml/kg body weight, based on day 7 of pregnancy. Aroclor 1254 was given at dosage levels of 0 (peanut oil only), 10, 50 and 100 mg/kg/day. Aroclor 1260 was given at dosage levels of 0 and 100 mg/kg/day. The females were allowed to deliver and the litters were observed through weaning.

Tissue preparation. Tissues were fixed in buffered 4% formaldehyde solution and stained with haematoxylin and eosin for microscopic examination. An Oil Red O stain on fixed frozen sections was used on occasional livers to demonstrate lipids.

Statistics and calculations

LD_{50} values were calculated by the method of Litchfield & Wilcoxon (1949). A *t* test was used for comparing the litter sizes, organ weights, body weights and haematological parameters of the treated groups with those of the control groups. Survival percentages of offspring were compared by the Mann–Whitney *U* test as proposed by Weil (1970). Food consumption values were arrived at by averaging the consumption of both males and females over the five periods in which food was measured.

RESULTS

LD_{50} studies on Aroclor 1254 and 1260

The single-dose oral LD_{50} for Aroclor 1254 in weanling male rats was 1295 mg/kg with 95% confidence limits of 1136–1476 mg/kg. The lowest lethal dose tested was 1200 mg/kg, and 1000 mg/kg produced mild diarrhoea. Signs of toxicity at lethal levels were diarrhoea, depression and salivation, with death occurring in 1–3 days.

For Aroclor 1260, the single-dose oral LD_{50} in weanling males was 1315 mg/kg with 95% confidence limits of 1174–1473 mg/kg. The lowest lethal dose tested was 1000 mg/kg, 800 mg/kg produced no signs of toxicity. Rats given lethal doses developed diarrhoea and depression, with death occurring in 1–7 days.

Under the same test conditions the estimated oral LD_{50} for Aroclor 1254 and 1260 in adult rats was 4–10 g/kg (Kimbrough *et al.* 1972).

The single-dose iv LD_{50} for Aroclor 1254 in adult female rats was 358 mg/kg with 95%

confidence limits of 328–390 mg/kg. The lowest lethal dose tested was 300 mg/kg; 250 mg/kg produced dyspnoea, depression, diarrhoea and salivation soon after dosing. Most rats died within 25–110 min, but at the highest dose (400 mg/kg), death occurred as soon as 5 min after dosing in some animals. Rats that survived appeared normal after 48 hr. In the control group, which received the formulation vehicle only, one rat developed mild diarrhoea.

Preliminary one-generation reproduction studies on Aroclor 1254 and 1260

The reproduction summary for the Aroclor 1254 feeding studies is presented in Table 1. Exposure for 67 days (at the time of pair-mating) to 500 ppm Aroclor 1254 (37·0 mg/kg/day) resulted in fewer litters, smaller litter sizes and 100% mortality by day 3 in the F_{1a} pups. The survival of offspring of the rats fed 100 ppm (7·2 mg/kg/day) for 67 and 186 days before mating was reduced, only 85·9 and 68·1% of the pups surviving to weaning in the F_{1a} and F_{1b} generations, respectively. Litter sizes from both matings were smaller, but the difference was not statistically significant. The mean body weight of the 100 ppm pups at weaning was 7·8 and 10 g less than that of the controls for the F_{1a} and F_{1b} generations, respectively. This difference was probably dose-related, as the treated litters were smaller, but a statistical evaluation was not done. In general the pups appeared small, but otherwise were normal at weaning.

Aroclor 1260 fed at a dietary level of 500 ppm (35·4 mg/kg/day) for 67 and 186 days prior to mating markedly reduced live-litter size and survival-to-weaning in the F_{1a} and F_{1b} generations (Table 2). No effect on reproduction was observed in rats fed 100 ppm (6·9 mg/kg/day) in either the F_{1a} or F_{1b} generations.

The F_0 rats in the one-generation studies were sacrificed after exposure for 8 months. Organ-weight analysis indicated that the liver was the primary organ affected. As reported in the previous paper, the livers in male rats receiving either Aroclor 1254 or 1260 at dietary levels of 100 and 500 ppm were heavier than in controls; this was also observed in females fed Aroclor 1254 at levels of 100 and 500 ppm or Aroclor 1260 at 500 ppm (Kimbrough *et al.* 1972). The testes-to-body weight ratios of F_0 rats fed Aroclor 1254 or 1260 at the 500 ppm level were greater than those of the controls ($P < 0·025$). The difference in actual weight of the testes, however, was not significant. This observation may reflect the reduced body-weight gain observed in all F_0 rats fed the 500 ppm level of both Aroclors. No difference in other organ weights was found.

After 8 months exposure, terminal haematological values in F_0 rats indicated a reduction in haemoglobin and haematocrit ($P < 0·005$) in both sexes fed 500 ppm Aroclor 1254 or 1260. The haematocrit was reduced in males ($P < 0·05$) on 100 ppm Aroclor 1260, but haemoglobin values were normal in this group. The total leucocyte count was normal in rats fed Aroclor 1260, but there was a shift in the differential count. The number of lymphocytes was increased in females ($P < 0·005$) fed 100 and 500 ppm Aroclor 1260 and in males ($P < 0·05$) fed 500 ppm. A corresponding decrease ($P < 0·05$) in polymorphonuclear leucocytes was observed. Differential values for rats fed Aroclor 1254 were normal, but an increase ($P < 0·001$) in total leucocytes was observed in the 500 ppm females.

Two-generation reproduction studies on Aroclor 1254

F_0 rats fed 100 ppm Aroclor 1254 (7·6 mg/kg/day) for 62 or 188 days before mating produced smaller litters than the controls in both the F_{1a} and F_{1b} generations (Table 1). Survival-to-weaning was not affected in the F_{1a} offspring. Only 73·6% of the 100 ppm F_{1b} pups

Table 1. *Reproduction and pup survival in groups of rats fed Aroclor 1254*

Generation	Dietary level (ppm)	Parental exposure (days)†	No. of females mated	No. of litters		Litter sizes§		Total pups/group				Survival (%)	Mean body weight at weaning (g)
								Born (found)		Alive at			
				Born‡	Weaned	At birth	At weaning	Dead	Alive	Day 3	Weaning		
F$_{1a}$	0‖	67	10	8	7	11·1	10·6	3	89	86	85	95·5	39·2
	100‖	67	10	9	8	9·4	8·1	6	85	78	73	85·9	31·4
	500‖	67	10	4 (2)	0	4·0*	—	5	8	0	0	0	—
	0	62	20	17	17	12·4	11·8	0	211	205	201	95·3	38·7
	20	62	20	19	19	11·7	11·5	3	222	220	219	98·6	38·3
	100	62	20	19	19	10·7*	10·3	10	204	199	196	96·1	32·9
	0	67	20	18	18	11·2	11·1	2	202	200	200	99·0	38·5
	1	67	20	16	15	9·2	9·1	3	147	145	145	98·6	42·3
	5	67	20	17	17	10·8	10·8	1	184	184	183	99·5	42·1
F$_{1b}$	0‖	186	10	7	7	11·6	11·6	1	81	81	81	100	37·9
	100‖	186	10	8	6	11·8	8·0	0	94	74	64	68·1**	27·9
	0	188	20	17	17	12·3	11·1	2	209	194	189	90·4	37·1
	20	188	20	18	18	10·4*	10·1	1	188	187	181	96·3	39·0
	100	188	20	20	19	9·5**	7·0	6	189	164	139	73·6	32·1
	0	201	20	18	18	12·3	11·0	6	222	211	198	89·2	40·7
	1	201	20	18	17	11·1	9·8	3	200	194	176	88·0	38·0
	5	201	20	20	19	11·1	10·2	2	222	218	203	91·4	38·9
F$_{2a}$	0	129	20	18	18	12·5	12·2	0	225	221	220	97·8	35·5
	20	129	20	17	17	10·6*	10·1	5	181	174	171	94·5	36·8
	100	129	20	7 (2)	4	7·2**	5·6	7	36	29	28	77·8**	35·2
	0	125	20	19	19	11·7	11·5	3	223	220	218	97·8	37·4
	1	125	20	15	15	11·7	11·5	0	175	174	173	98·9	37·2
	5	125	19	17	17	12·1	11·7	1	205	199	198	96·6	35·7
F$_{2b}$	0	274	20	17	16	12·7	11·3	4	216	201	191	88·4	31·9
	20	274	20	12	12	9·6**	8·5	6	115	103	102	88·7	36·1
	100	274	20	4 (2)	2	3·5**	3·5	7	7	7	7	100·0	38·3

† Conception to mating for the parents of the F$_{2a}$ and F$_{2b}$ generations.

‡ Numbers in parentheses indicate numbers of litters in which no live offspring were found.

§ Number of live offspring/live litter born.

‖ One-generation study.

Values marked with asterisks differ significantly from the control value: * $P < 0·05$; ** $P < 0·005$.

survived to weaning, but this was not statistically significant ($P = 0{\cdot}058$). Because of the poor condition of a number of the 100 ppm F_{1b} pups, all were held for 30-days post-weaning observation. Fifteen of these pups died within 30 days of weaning. The mean body weights of the 100 ppm F_1 generation pups from either mating was about 6 g less than the controls at weaning. This is probably significant, since the treated litters contained fewer pups. A reduction in litter size was also observed in the F_{1b} generation at 20 ppm (1·5 mg kg/day).

F_{1b} rats bred after exposure for 129 or 274 days (conception to mating) to 20 or 100 ppm had smaller litters than the controls (F_{2a} and F_{2b} generations). A marked decrease in mating performance was observed in the 100 ppm group, in which only seven and four litters were born in the F_{2a} and F_{2b} generations, respectively. In each case two litters contained no viable offspring when first observed. The data also suggest a decrease in mating performance at 20 ppm, at which level only 12 F_{2b} litters were produced. Survival-to-weaning was reduced in the 100 ppm F_{2a} offspring. All 100 ppm F_{2b} offspring survived to weaning, but this is of little significance since only seven pups were found alive. No effect on reproduction was observed in rats fed 5 ppm (0·32 mg/kg/day) or 1 ppm (0·06 mg/kg/day) through two generations.

Although reproduction was not affected at lower dietary levels of PCBs, an increase in liver weight in 21-day-old weanlings was found at all levels tested (Table 2). At 5 ppm or higher, the liver-to-body weight ratios of weanlings were increased in both sexes in both

Table 2. *Mean liver weights of 21-day-old rats from parents fed Aroclor 1254 or 1260*

			Liver weight			
			Males		Females	
Aroclor	Generation	Dietary level (ppm)	g	% of body weight	g	% of body weight
1254	F_{1a}	0	1·40	3·69	1·38	3·83
		1	1·81*	4·01*	1·63	3·91
		5	1·92*	4·34*	1·86*	4·43*
	F_{1b}	0	1·58	3·69	1·58	3·84
		1	1·54	3·94*	1·49	3·92
		5	1·77	4·63*	1·72	4·75*
	F_{2a}	0	1·37	3·81	1·28	3·74
		20	2·26*	5·78*	2·23*	6·08*
		100	2·63*	6·97*	2·67*	7·10*
	F_{2a}	0	1·63	3·78	1·42	3·63
		1	1·49	3·74	1·36	3·69
		5	1·56	4·23*	1·38	4·09*
1260	F_{1a}	0	1·54	3·66	1·57	3·92
		5	1·73	4·18*	1·70	4·39*
	F_{1b}	0	1·59	3·73	1·55	3·93
		20	2·06*	5·04*	2·14*	5·18*
		100	2·59*	6·33*	2·59*	6·48*
	F_{1b}	0	1·35	3·59	1·29	3·70
		5	1·58	4·01*	1·52*	4·07*
	F_{2a}	0	1·65	3·82	1·64	4·02
		20	2·06*	5·21*	1·94	5·06*
		100	2·50*	6·12*	2·49*	6·85*
	F_{2a}	0	1·41	3·85	1·36	3·87
		5	1·66*	4·21*	1·60*	4·25*

Values marked with an asterisk differ significantly from the control value: * $P < 0{\cdot}05$.

the F_1 and F_2 generations, but at 1 ppm the liver enlargement was observed only in the F_{1a} and F_{1b} male weanlings. No liver-weight increase was found in either sex of the 1 ppm F_{2a} weanlings.

In 10-month-old F_{1b} rats, an increase in liver-to-body weight ratios was observed in both sexes at 100 ppm, but at 20 ppm it was seen only in the males (Table 3). At 5 ppm or less no increase in liver weight was found in 6-month-old F_{1b} rats or in F_0 rats exposed for 10 months. Both sexes of F_0 rats fed 20 ppm or more Aroclor 1254 for 8 months had enlarged livers (Kimbrough *et al.* 1972). The testes-to-body weight ratios were increased ($P < 0{\cdot}001$) in 100 ppm F_{1b} adult males. Body-weight gains of adults in all the test groups were comparable to those of the controls except in the F_{1b} females at 100 ppm, which gained less weight than the controls (Table 3). Reduced haemoglobin ($P < 0{\cdot}005$) and haematocrit ($P < 0{\cdot}025$) were observed in female F_{1b} adults at 100 ppm, while F_{1b} males at this level had reduced ($P < 0{\cdot}05$) haemoglobin only. Haematological values at lower levels were normal. No dose-related signs of toxicity were observed in adult F_0 or F_{1b} rats fed 100 ppm or less.

Two-generation reproduction study on Aroclor 1260

No effect on reproduction was observed in rats fed Aroclor 1260 at levels of 100, 20 or 5 ppm (7·4, 1·5 and 0·39 mg/kg/day) through two generations (Table 4). The liver-to-body weight ratios of 21-day-old pups were increased at all dietary levels (Table 2). This effect was observed at the lowest level tested (5 ppm) in all generations.

An increase in liver-to-body weight ratios was observed at all dietary levels in F_{1b} males between 5 and 7 months old, but only at 100 ppm in the F_{1b} females (Table 3). After 8 months exposure, an increase in liver weight was observed at 20 and 100 ppm in F_0 males but not in females (Kimbrough *et al.* 1972). No effect on liver weight was found in either sex of F_0 rats fed 5 ppm for 8 months. The testes-to-body weight ratios of the 100 ppm F_{1b} males were increased ($P < 0{\cdot}05$); at 20 ppm the increase was not significant. Body-weight gain in all test groups was comparable to the control groups. Haematological values were normal in the 20 ppm F_0 adults and in F_{1b} adults at 100 ppm or less. No signs of toxicity were observed in any of the test animals.

Post-implantation exposure studies on Aroclor 1254 and 1260

The data on reproduction and survival of offspring from females dosed during organogenesis is summarized in Table 5. Nine oral doses of Aroclor 1254 (100 mg/kg/day) given on days 7–15 of gestation resulted in a decrease in the survival of the pups. At this dose only 30·1% of the offspring of the treated group survived to weaning, compared with 98·2% in the control group, and the mean body weight of the test pups was 7·1 g less than that of the controls at weaning. No effect was observed in the groups receiving 50 mg/kg/day or less.

No effect on reproduction or survival was observed with Aroclor 1260 given at a rate of 100 mg/kg/day.

Summary of pathology after treatment with Aroclor 1254 and 1260

A detailed account of the liver pathology in the F_0 rats fed Aroclor 1254 or 1260 at 20 ppm or higher dietary levels has been published (Kimbrough *et al.* 1972). Of the rats considered in the current report, similar changes were observed in the liver of adult rats, particularly in the F_{1b} generations at the higher dietary levels. The incidence of the various

Table 3. *Body weight, liver weight and liver pathology of F_0 and F_{1b} adult rats fed Aroclor 1254 or 1260*

Generation	Sex	Exposure (days)	Dietary level (ppm)	Terminal body weight (g)	Liver weight: g	Liver weight: % of body weight	No. of livers examined	Liver pathology (no. of livers affected): Enlarged hepatocytes	Inclusions	Foamy cytoplasm	Pigment	Fibrous strands	Adenofibrosis	Nodules
Aroclor 1254														
F_0	M	310	0	601	15·22	2·53	10	0	0	0	0	0	0	0
			1	602	15·88	2·60	10	0	0	0	0	0	0	0
			5	614	15·37	2·51	10	0	0	1	2	0	0	0
	F	313	0	370	10·34	2·80	10	0	0	0	1	0	0	0
			1	358	10·14	2·84	10	1	0	0	4	0	0	0
			5	362	10·66	2·94	10	4	1	0	3	0	0	0
F_{1b}	M	328	0	588	14·51	2·47	10	0	0	0	0	0	0	0
			20	602	16·11*	2·67*	8	8	3	7	1	0	0	0
			100	554	19·43*	3·54*	10	10	5	7	4	2	3	1
	F	328	0	349	9·92	2·85	10	0	0	0	0	0	0	0
			20	340	9·41	2·78	7	7	2	5	7	0	0	3
			100	300*	10·53	3·60*	10†	10	2	8	9	3	5	7
F_{1b}	M	190	0	475	11·13	2·34	10	0	0	0	0	0	0	0
			1	453	11·14	2·44	10	0	0	0	0	1	0	0
			5	495	12·19	2·46	10	2	0	2	0	1	0	0
	F	190	0	287	8·27	2·87	10	0	0	0	0	0	0	0
			1	294	8·44	2·86	10	1	1	0	0	4	0	0
			5	299	8·92	2·98	10	1	0	0	0	1	0	0
Aroclor 1260														
F_0	M	250	0	563	14·45	2·57	10	1	0	0	0	0	0	0
			5	567	15·06	2·65	10	3	0	2	0	0	0	0
	F	250	0	322	10·09	3·13	10	0	0	0	0	0	0	0
			5	331	10·80	3·25	10	1	0	0	0	0	0	0
F_{1b}	M	179	0	472	12·68	2·68	10	0	0	0	0	0	0	0
			20	494	14·72*	2·99*	10	8	5	4	0	0	0	0
			100	509	16·63*	3·27*	10	10	10	3	0	0	0	1
	F	179	0	297	9·66	3·27	10	0	0	0	0	0	0	0
			20	304	10·30	3·40	10	6	0	0	0	0	0	0
			100	304	11·14*	3·70*	10	9	0	1	5	2	1	2
F_{1b}	M	217	0	492	11·97	2·43	10	0	0	0	0	0	0	0
			5	505	13·16	2·60*	10	7	2	2	1	0	0	0
	F	217	0	279	7·93	2·84	10	0	0	0	0	0	0	0
			5	286	8·49	2·96	10	2	0	0	0	0	0	0

† Necrosis in two livers.
Values marked with an asterisk differ significantly from control group: $^*P < 0{\cdot}025$.

5	286	8·49	2·56	10	2	0	0	0	0	0	0

† Necrosis in two livers.
Values marked with an asterisk differ significantly from control group: $^*P < 0·025$.

Table 4. *Reproduction and pup survival in groups of rats fed Aroclor 1260*

Generation	Dietary level (ppm)	Parental exposure (days)†	No. of females mated	No. of litters		Litter size‡		Total pups/group				Survival (%)	Mean body weight at weaning (g)
								Born (found)		Alive at			
				Born	Weaned	At birth	At weaning	Dead	Alive	Day 3	Weaning		
F_{1a}	0§	67	10	9	9	12·3	12·2	2	111	111	110	99·1	37·0
	100§	67	10	8	8	11·6	11·6	0	93	93	93	100·0	[illegible]
	500§	67	8	8	3	8·5**	3·3	10	68	55	26	38·2*	36·3
	0	68	20	19	19	11·7	11·2	2	223	217	213	95·5	40·9
	20	68	20	17	17	11·8	11·3	0	201	193	191	95·0	39·8
	100	68	20	17	17	11·6	11·2	1	197	192	191	97·0	38·0
	0	71	20	19	19	11·7	11·4	0	222	218	216	97·3	33·8
	5	71	20	18	18	12·1	11·8	1	217	213	212	97·7	38·5
F_{1b}	0§	186	10	9	8	12·1	10·3	1	109	96	93	85·3	40·9
	100§	186	9	5	5	11·0	10·8	0	55	54	54	98·2	36·2
	500§	186	8	6	2	6·7**	2·3	1	40	38	14	35·0	40·7
	0	187	20	17	17	11·1	11·0	6	189	189	187	98·9	40·8
	20	187	20	16	16	11·9	11·4	1	191	191	183	95·8	41·0
	100	187	20	13	13	10·4	10·1	5	135	131	131	97·0	39·2
	0	188	20	17	16	10·3	9·2	10	175	160	156	89·1	36·1
	5	188	19	16	15	11·2	10·6	2	179	171	170	95·0	39·3
F_{2a}	0	128	20	18	18	10·9	10·3	1	197	187	185	93·9	40·3
	20	128	20	19	19	11·3	11·1	1	215	213	211	98·1	38·7
	100	128	20	20	20	10·1	9·9	1	201	200	197	98·0	38·6
	0	127	20	13	13	11·8	11·6	1	153	152	151	98·7	34·8
	5	127	20	18	18	11·8	11·6	0	213	212	209	98·1	38·5

† Conception to mating for parents of the F_{2a} generation.
‡ Numbers of live offspring/live litter born.
§ One-generation study.
Values marked with asterisks differ significantly from the control value: $^*P < 0·025$; $^{**}P < 0·001$.

Table 5. Reproduction and survival of pups from dams dosed orally with Aroclor 1254 or 1260 on days 7–15 of pregnancy

Dose (mg/kg/day)	No. of females treated	No. of litters		Litter size†		Total pups/group				Survival (%)	Mean body weight at weaning (g)
						Born (found)		Alive at			
		Born	Weaned	At birth	At weaning	Dead	Alive	Day 3	Weaning		
					Aroclor 1254						
0	9	9	9	12·3	12·1	0	111	110	109	98·2	37·9
100	9	7	5	11·9	3·6	8	83	64	25	30·1*	30·8
0	10	10	10	11·9	11·8	0	119	119	118	99·1	40·6
10	9	9	9	12·1	12·0	0	109	109	108	99·1	37·6
50	10	10	10	13·4	12·7	0	134	131	127	94·8	30·5
					Aroclor 1260						
0	12	12	12	12·0	11·5	4	144	140	138	95·8	33·9
100	12	12	11	11·7	10·3	10	140	135	124	88·6	33·9

† No. of live offspring/litter born.
The value marked with an asterisk differs significantly from the control value: $^{*}P < 0{\cdot}001$.

changes is given in Table 3. Enlargement of the liver cells, involving primarily an increase of the cytoplasm surrounding the nucleus, was the most consistent finding and was occasionally observed at the 5 and 1 ppm dietary levels. Inclusions within the cytoplasm, which have also been referred to by some authors (Bennett, Drinker & Warren, 1938; Miller, 1944) as hyaline bodies, were observed more frequently in the males than in the females and were only occasionally noticed at the lower dietary levels. At 5 and 1 ppm the cytoplasm of many liver cells was vacuolated or foamy. According to the studies reported previously (Kimbrough *et al.* 1972), these changes were indicative of lipid accumulation. They were more pronounced at the higher dietary levels of 20 and 100 ppm. Adenofibrosis, which was also described in the previous paper, was observed only at the 100 ppm dietary level.

Two additional findings made in adult F_{1b} rats had not been observed previously in the F_0 rats. One was the occasional occurrence of strands of fibrous tissue which traversed the liver. In addition, hepatic nodules measuring up to 0·5 cm in diameter were observed in occasional livers of F_{1b} adults fed 100 ppm Aroclor 1254 or 1260. In addition, of the seven livers studied from F_{1b} adults fed 20 ppm Aroclor 1254, three showed one or more hepatic nodules. The hepatic nodules consisted of enlarged liver cells, which formed well-organized hepatic cords. The cytoplasm of many of these liver cells, which often stained somewhat lighter than the surrounding liver tissue, contained one or more inclusions. The nuclei of the liver cells within the hepatic nodules appeared normal (Fig. 1).

Apart from the findings in the livers, occasional rats of the control as well as the exposed groups showed mild-to-moderate chronic pyelonephritis. The incidence in the exposed group did not differ from that of the controls. One and two female rats fed 20 and 100 ppm Aroclor 1260, respectively, and two male rats fed 100 ppm Aroclor 1254 showed hyperplasia of the thyroid. Hyperplasia of the thyroid was not observed in the controls, but occasionally it does occur spontaneously in our rats. Whether this low incidence of hyperplasia of the thyroid is related to the consumption of Aroclor cannot be established with certainty from this study. No other morphological changes were observed in any of the other organs, including the testes.

The livers of F_{1b} weanlings from the group given the 20 ppm level of Aroclor 1254 showed slight enlargement of the liver cells but no other morphological changes. The livers of ten F_{1b} weanling rats at the 100 ppm level of Aroclor 1254 showed marked enlargement of the liver cells, which had either foamy or vacuolated cytoplasm. At 20 ppm, the livers of ten males and ten female F_{2a} weanling rats showed greatly enlarged liver cells, and the cytoplasm was vacuolated in most instances. Oil Red O stain on a few of these livers showed that vacuolization was due to lipid accumulation. Similar findings were made in four male and four female F_{2a} weanlings at the 100 ppm level of Aroclor 1254.

The livers of ten male and ten female F_{1b} weanling offspring from dams fed 20 ppm Aroclor 1260 had normal livers on microscopic examination, while those at 100 ppm showed enlarged and vacuolated hepatocytes. The livers of ten of 20 F_{2a} weanlings at the 20 ppm level of Aroclor 1260 and 17 of 19 livers of F_{2a} weanlings at the 100 ppm level showed enlarged cells and vacuolated cytoplasm.

At the lower dietary levels of both Aroclor 1254 and 1260 (5 ppm or less), livers of weanling rats were only occasionally examined in the different generations. The only microscopic change observed consisted of slightly enlarged hepatocytes in some of the livers.

DISCUSSION

The results of our studies confirm the capability of some commercial PCB mixtures to alter reproductive processes in mammals. Our data are in general agreement with those of Keplinger, Fancher & Calandra (1971), who observed effects on mating performance and/or pup survival in rats fed 100 ppm Aroclor 1242 or 1254, but found no effect at the same dietary level of Aroclor 1260. In our study, 20 ppm Aroclor 1254 affected litter size and probably decreased mating performance in the second mating of the F_{1b} rats. The trend suggested the possibility that even lower levels might affect reproduction in subsequent generations, but M. L. Keplinger (personal communication 1973) found no effect through three generations in rats fed 10 ppm Aroclor 1254. Reproductive failure in mink exposed to PCBs has also been reported (Ringer *et al.* 1972). Although actual PCB consumption on a mg/kg basis was not reported, the mink is apparently more sensitive to PCB exposure than the rat, since levels of 1 and 5 ppm Aroclor 1254 affected reproduction after only 4 months' exposure. In the present study in rats, fewer pups per litter were observed at dietary levels of 500 ppm Aroclor 1260 and of 20 ppm or more of Aroclor 1254. Reported litter sizes are based on the pups found, but since it is common for the dam to eat dead or defective offspring, the reduced litter size probably reflects decreased fertility and/or increased perinatal and foetal mortality.

Aroclor 1254 (100 mg/kg/day) given to pregnant rats during gestation produced no effect on the viability or morphology of foetuses examined on day 22 of gestation (Villeneuve, Grant, Khera, Clegg, Baer & Phillips, 1971). In the present study under a similar dosing regimen, dams dosed orally during gestation with Aroclor 1254 (100 mg/kg/day) gave birth to litters that were grossly normal, but only 30·1% of the pups survived to weaning. Since PCBs are excreted in milk (Curley, Burse & Grim, 1973; Fries, 1972), it is possible that PCB ingestion via the milk contributed to the increased mortality of pups observed in both the post-implantation and dietary-exposure studies. The milk of rats 11 days after the last of nine daily doses of Aroclor 1254 (50 mg/kg/day) contained 66 ppm of PCB-derived material (Curley *et al.* 1973). Kimbrough *et al.* (1972) estimated the oral LD_{50} for Aroclor 1254 and 1260 at 4–10 g/kg in adult rats. From the oral LD_{50} values in weanlings in the present study (about 1300 mg/kg), it is evident that the Aroclor mixtures tested are considerably more toxic to immature rats than to adults. Although we have no acute toxicity data for PCBs in neonates, nor figures on their milk consumption, it is possible that lacteal exposure could approach a toxic level in suckling rats at the higher exposure levels.

In both the dietary and post-implantation studies, direct PCB exposure occurred *in utero*, since PCBs have been shown to cross the placenta (Curley *et al.* 1973; Grant, Villeneuve, McCully & Phillips, 1971). PCBs have been reported in eggs (Peakall, 1971) and embryonic mortality has been observed in birds treated experimentally with commercial PCB mixtures (Dahlgren *et al.* 1972; Peakall, Lincer & Bloom, 1972). Dahlgren *et al.* (1972) also reported a decrease in survival and a depression of weight gain in 6-wk-old pheasant chicks from hens treated with Aroclor 1254. In the present studies, similar effects were observed in young rats at the higher exposure levels of Aroclor 1254. Besides a reduction in observed litter size, there was additional evidence of late foetal mortality in the group given 100 ppm Aroclor 1254, no live offspring being found in four of the F_2 litters. Thus it is apparent that some PCB mixtures affect the embryo in both mammalian and avian systems, although the mechanism of this effect remains undefined.

Although the suckling rats ate some of the Aroclor-treated diet for 4–5 days before weaning, it is probable that the observed increase in liver weight in 21-day-old rats was

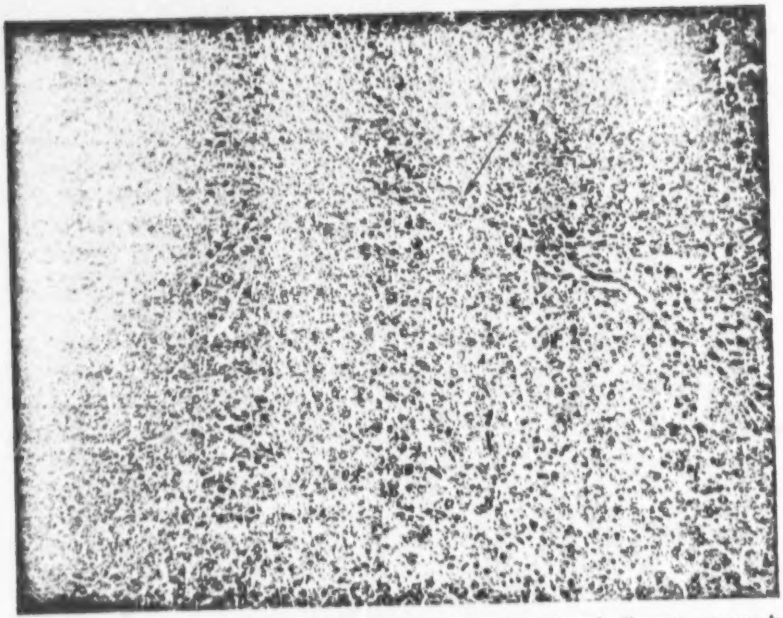

Fig. 1. Section of rat liver, showing a hepatic nodule measuring 0·2 cm in diameter surrounded by normal liver cells (arrow at margin of hepatic nodule). The hepatocytes within the nodule are larger than those of the surrounding tissue and stain less well. Haematoxylin and eosin × 100.

due to PCB exposure via the mothers' milk. Curley *et al.* (1973) also observed a liver-weight increase in weanlings from dams dosed only during gestation. When Aroclor 1254 was given during pregnancy to rabbits, maternal liver weight was increased but not foetal liver weight on day 29 of gestation (Villeneuve *et al.* 1971), thus eliminating the possibility that placental transfer of PCB was the cause of the liver-weight increase seen in weanlings. When adult rats of the F_{1b} generation were killed after dietary exposure for 5–6 months, liver weights at the lower dietary levels were comparable to those in the controls except in males given 5 ppm Aroclor 1260. Presumably once exposure via the milk ceased, the livers returned to normal in spite of a continued low dietary intake of PCB. At a dietary level of 1 ppm Aroclor 1254, only the male weanlings of the F_{1a} and F_{1b} generations had enlarged livers. The absence of this effect in the F_{2a} weanlings is inconsistent. One explanation could be the credibility of the 1 ppm formulation. Problems inherent in consistently reproducing a 1 ppm formulation, as well as contamination of the basal diet with PCBs, are possible sources of error. Over the last 18 months this laboratory has analysed the basal diet for PCB contamination at 6-wk intervals. Most values have been less than 0·1 ppm, but occasionally values of up to 0·5 ppm have been found. The latter level of contamination would introduce a 50% error in a 1 ppm formulation. It is likely that 1 ppm in the parental diet is very near a threshold effect level for the observed liver-weight increase in weanling rats.

At the lower dietary levels, adverse effects were not observed in weanling rats, which exhibited only an increase in liver weight. However, Aroclor 1254 fed to 1-month-old rats at the 100 ppm dietary level (approximately 7·9 mg/kg/day) increased cytochrome *P*-450 and liver weight after 2 days and microsomal protein after 3 days, while a single oral dose of 10 mg/kg increased cytochrome *P*-450 after 24 hr (Goldstein, Hickman & Jue, 1973). A 50–70% increase in nitroreductase activity was observed with Aroclor 1254 and 1260 fed to rats at a dietary level of 0·5 ppm for 4 wk (Litterst, Farber, Baker & Van Loon, 1972). In view of these reports it is very probable that offspring in the reproduction studies, even those involving the lowest levels of PCBs, were subject to inductive effects.

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L'effet des biphényles polychlorurés sur la reproduction du rat

Résumé—On a étudié la reproduction, la pathologie et les indices d'intoxication aiguë chez des rats auxquels on administrait l'un ou l'autre des biphényles polychlorurés Aroclor 1254 et Aroclor 1260. Les rats qui recevaient l'Aroclor 1254 à raison de 20 ppm ou plus du régime avaient des nichées moins nombreuses que les animaux témoins aux générations F_{1b} et F_2. La dose de 100 ppm d'Aroclor 1254 a fait augmenter la mortalité dans la génération F_{1b} et fait nettement diminuer les performances d'accouplement des adultes de cette génération. Administré à raison de 500 ppm du régime, l'Aroclor 1260 a fait diminuer l'importance des nichées et le taux de survie des nichées F_1.

Des taux de 5 ppm d'Aroclor 1254 et de 100 ppm d'Aroclor 1260 sont restés sans effet sur la reproduction des rats soumis à ces régimes pendant deux générations. Le poids du foie a augmenté chez les mâles F_1 âgés de 21 jours au régime à 1 ppm d'Aroclor 1254 et chez les mâles et femelles F_1 et F_2 sevrés des groupes à 5 ppm ou plus d'Aroclor 1254 ou 1260. Des modifications histologiques du foie et des augmentations du poids de cet organe ont été observées chez les rats adultes qui recevaient les plus fortes doses. Des femelles gravides qui avaient reçu 100 mg d'Aroclor 1254/kg de poids vif et par jour du 7ème au 15ème jour de gestation ont mis bas des nichées à peu près normales, mais seulement 30,1% de ces jeunes ont survécu au sevrage. La reproduction et le taux de survie des jeunes n'ont pas été influencés par les doses de 50 mg d'Aroclor 1254/kg/jour et de 100 mg d'Aroclor 1260/kg/jour. Les valeurs DL_{50} orales chez les rats mâles âgés de 3–4 semaines étaient de 1295 mg/kg d'Aroclor 1254 et de 1315 mg/kg d'Aroclor 1260. La LD_{50} intraveineuse d'Aroclor 1254 était de 358 mg/kg chez les femelles adultes.

Der Einfluss von polychlorierten Biphenylen auf die Fortpflanzung der Ratte

Zusammenfassung—Vermehrung, Pathologie und akute Toxizität wurden an Sherman-Ratten untersucht, die den polychlorierten Biphenylen Aroclor 1254 und Aroclor 1260 ausgesetzt wurden. Ratten, die Aroclor 1254 in Konzentrationen von 20 ppm oder darüber im Futter erhielten, hatten weniger Junge je Wurf als die Kontrolltiere in den F_{1b}- und F_2-Generationen. Die Konzentration 100 ppm von Aroclor 1254 erhöhte die Sterblichkeit der F_{1b}-Nachkommen und setzte deutlich die Paarungsleistung der F_{1b}-Erwachsenen herab. Die Konzentration von 500 ppm Aroclor 1260 im Futter verminderte die Grösse der Würfe und verminderte das Überleben in den F_1-Würfen. Die Konzentrationen von 5 ppm Aroclor 1254 und 100 ppm Aroclor 1260 hatten keinen Einfluss auf die Fortpflanzung von Ratten, die ihnen über zwei Generationen ausgesetzt waren. Das Lebergewicht war bei 21 Tage alten männlichen abgesetzten F_1-Tieren bei der Konzentration von 1 ppm Aroclor 1254 und bei beiden Geschlechtern von abgesetzten F_1- und F_2-Jungen bei 5 ppm oder höheren Konzentrationen von Aroclor 1254 und 1260 erhöht. Histologische Veränderungen der Leber und erhöhtes Lebergewicht wurden bei erwachsenen Ratten beobachtet, welche die höheren Konzentrationen erhielten. Trächtige Ratten, die Aroclor 1254 in der Konzentration 100 mg/kg/Tag vom 7. bis 15. Tag der Trächtigkeit erhielten, brachten äusserlich normale Würfe hervor, aber nur 30,1% der Jungen überlebten bis zum Absetzen. Die Fortpflanzung und das Überleben der Nachkommen wurden bei Dosierungen von 50 mg Aroclor 1254/kg/Tag oder 100 mg Aroclor 1260/kg/Tag nicht beeinflusst. Die oralen LD_{50}-Werte bei 3–4 Wochen alten männlichen Ratten betrugen 1295 und 1315 mg/kg bei Aroclor 1254 bzw. 1260. Die iv LD_{50} für Aroclor 1254 bei erwachsenen weiblichen Tieren betrug 358 mg/kg.

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Visual Effects Accompanying TEPP-Induced Miosis

PUBLICATION

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Pilots and others have long recognized the miosis which is frequently a consequence of spraying tetraethylpyrophosphate (TEPP) dust by aircraft. This effect of TEPP often occurs without symptoms referable to any part of the body except the eyes. Parathion (O,O-diethyl O-para-nitrophenyl thiophosphate) may also produce miosis, but it does so less frequently, and when it does the eye signs and symptoms are usually accompanied by general, systemic illness. The reason for the difference in clinical effect is not entirely clear but probably involves, among other things, the difference in molecular weight and vapor pressure of the two compounds.

Various flying accidents have been blamed on the effects of organic phosphates on pilots.* Two complaints which are occasionally made by the pilots themselves involve an inability to judge distance and a sensation that things are darker than the time of day, the season, and the weather justify.

A study in 1953 of the visual fields of 15 pilots and 2 other persons before, during, and after occupational exposure to TEPP did not reveal any meaningful change. However, only one pilot in this series was available for study of visual fields at a time when he actually had miosis. A much greater diversity of tests conducted during miosis seemed to be required to explain the reported disturbances of function.

The dosage level in the eye required to contract the pupil to 1 mm. diameter was worked out in an intensive study of one volunteer at three dosage levels. The study served to establish techniques for conducting a battery of ophthalmological tests. Instillation of 1 drop (about 0.01 cc.) of 0.0125% TEPP and later of 1 drop of 0.05% TEPP produced no measurable effect. Instillation of 2 drops (about 0.02 cc.) of 0.05% TEPP produced slight miosis and a slight increase of near accommodation. Maximal miosis was produced by 2 drops of 0.1% TEPP, the pupils being fixed at 1 mm. diameter. A fourth drop did not cause further miosis or other observed change. Visual acuity (Snellen), peripheral fields (Ferree-Rand simplified

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* References 1 and 4.

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perimeter), depth perception (Howard-Dolman), color vision (Ishihara), and subjective perception all remained within normal limits. The near point as measured by the Prince Rule moved from a predosage distance of about 13 cm. to 3.8 cm. for the right eye and 4.5 cm. for the left eye.

The next logical course of investigation was to induce miosis in a number of pilot-volunteers. Six men with no previous exposure during the spray season in which the tests were made were selected from a group of commercial dusting pilots and their occupational associates by screening with a medical and ophthalmologic history followed by medical examinations.

The TEPP used in this experiment consisted of 40% tetraethylpyrophosphate and 60% related organic phosphates. Each drop (0.01 ml.) of 0.1% technical (40%) TEPP contained 0.009 mg. of technical TEPP in peanut oil. The maximum dose (2 drops of 0.1% into each eye) totaled 0.036 mg. For the average body weight of 83 kg., the maximum dosage rate was 0.0004 mg/kg.

For each dosage level, the volunteers were given batteries of tests at intervals of one hour. About one week elapsed between the different TEPP dosages. With the minor exceptions noted, each battery of tests consisted of the following 23 tests.

The first group of tests in each battery was performed with the Ortho-Rater † for far vision—(1) vertical phoria; (2) lateral phoria; (3, 4, 5) acuity, eyes together and separately; (6) binocular depth perception; (7) color vision—and for near vision—(8, 9, 10) acuity, eyes together and separately; (11) vertical phoria; (12) lateral phoria. A transilluminated Snellen chart and associated rheostat ‡ were used to make the following tests: (13) Bilateral acuity. (This was checked at the 20/20 level on all candidates with full power load in the chart-lamp circuit. Tests of more critical acuity were not in order because subsequent threshold tests required the use of the 20/20 test line, which was the only one homogeneously illuminated at various intensities.) (14) Threshold of light intensity required for reading. (Following the test of bilateral acuity, the light intensity was turned down by means of the rheostat until the volunteer could not distinguish the letters and then turned up until he could. The reading in arbitrary units, 135 for the entire scale, was recorded.) (15) Threshold of light intensity required for perception. (For the tests at the highest dosage level, 2 drops of 0.1% TEPP, the rheostat reading was also recorded at the first point at which the subject could distinguish the faint pinkish light in the chart, i.e., the threshold of minimum light perception. As the power delivered by the rheostat to the light circuit was reduced, the quality of the light changed from incandescent white to orange and then to a faint pinkish glow before disappearing entirely.) The Prince Rule and No. 3 Jaeger print card were used to test (16) accommodation for near vision. The Snellen chart and appropriate lenses held in the usual test frame were used to test (17) accommodation for far vision in respect to positive lenses; (18) accommodation for far vision in respect to negative lenses. A camera was used to record (19, 20) pupil size and reaction to light. (The eye was photographed in a darkroom with normal lighting. After adaptation in the dark for five minutes, the eye was again photographed.) The Hare perimeter was used to determine (21) field of vision. (Readings were taken for white on the vertical and horizontal axes of the perimeter.) The Schiøtz tonometer was used to measure (22) ocular tension. Blood was drawn from the ear to determine

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† Bausch and Lomb Optical Company, Professional Model.
‡ Fisher Scientific Powerstat, Type 116, output range 135 volts.

(23) plasma and red blood cell cholinesterase levels, by use of a modification of the Michel electrometric procedure.[8] (Blood was drawn on two occasions before any TEPP was instilled into the eyes and again at the end of the last battery of tests at each dosage level.)

The results of the study are summarized in Table 1. Where there were no notable differences in the two eyes, only data for the left eye have been given in the Table. Some tests which gave negative or normal results at all dosage levels have been omitted from the Table.

TABLE 1.—*Selected Ophthalmologic Measurements of Certain Functions of the Eyes of Pilots into Which TEPP Had Been Instilled**

Function	Condition of Test	Unit of Measure	Eye†	Dosage Level and Time After Dosage					
				1 Drop Peanut Oil (Control)		2 Drops 0.1% TEPP			
				Before Dose	After 1 Hr.	Before Dose	1 Hr.	2 Hr.	3 Hr.
Diameter of pupil	Illuminated Dark	Mm. Mm.	L L	5.4 6.7	4.6 6.6	4.5 5.5	1.9 1.9	1.9 1.9	1.8 2.0
Acuity	Minimum light to read Snellen 20/20	Rheostat units	B	33.3	38.2	29.7	33.5	32.7	33.8
Light perception	Minimum light (pink)	Rheostat units	B			12.9	17.8	17.2	16.4
Accommodation	Prince Rule Snellen chart	Cm. Pos. diopters Neg. diopters	L B B	13.3 1.89 2.93	13.3 1.82 3.17	12.3 1.92 3.71	6.1 2.25 5.96	6.8 2.46 6.66	7.0 2.42 7.08
Acuity	Ortho-Rater, far vision	Ortho-Rater scoring units	B R L	10.8 11.3 11.3	11.8 12.3 11.5	11.8 11.4 12.7	9.5 9.3 9.8	10.2 9.7 10.3	10.0 9.5 10.7
	Ortho-Rater, near vision	Same	B R L	11.8 12.2 11.3	11.8 11.0 12.0	11.8 12.5 11.8	10.5 10.0 9.7	10.3 10.0 10.3	10.0 9.7 10.2
Depth perception	Ortho-Rater	Ortho-Rater units	B	6.7	5.3	6.5	6.2	5.8	6.3
Visual fields	Hare perimeter, horizontal Hare perimeter, vertical	Degrees subtended Same	L L	135.7 109.0	138.0 111.7	133.9 108.3	127.8 103.2	133.0 100.6	128.3 99.2
Ocular tension	Schiøtz tonometer	Mm. of Hg	R L		19.2 17.7				18.5 14.3

* Mean values from six subjects.
† Left eye, right eye, or both eyes, as indicated.

The production of maximal miosis, at least to the point of fixing the pupils, was considered one of the requisites of this study. Fixation of the pupils with concomitant effects was obtained consistently only with 2 drops of 0.1% TEPP spaced 30 minutes apart and not with a single drop.

Subjectively the maximum dosage caused mild headaches, sensations of pressure in the eyeballs or over the frons, slight burning of the lids, "running" or "stopped-up" nose, and a sensation that "things were getting darker." These symptoms passed their peak during the first or second hour. Four of the pilots stated, upon self-inspection in the mirror, that their pupils were not as small following the experimental exposures as they were following occupational exposure in the summer. One pilot believed that the experimental miosis was as severe as he had ever experienced in applying TEPP by airplane. The sixth man had had insufficient prior exposure to make a significant comparison.

At the highest dosage level, the subjective complaints of reduction of light perception were corroborated by the Snellen chart light-threshold test and also by the minimum perception of light in the same back-illuminated chart.

The Prince Rule test showed greatest accommodation for near vision at the one-hour observation battery (30 minutes after the second drop). The lens test for far accommodation revealed a gradually increasing accommodation, particularly to negative lenses, at least until the third hour. One of the subjects was tested at the fifth hour also and was found to have undergone no further change in this respect. The changes in accommodation may be accounted for, at least in part, on a physical basis. The accommodation for near vision and for far vision with negative lenses is associated with the increased convexity of the lens of the eye as well as with the greater depth of focus which accompanies constriction of the pupil. The accommodation for far vision with positive lenses can be explained by the greater depth of focus which accompanies constriction of the pupil

According to the Snellen measurement at the usual illumination, no change of acuity occurred at any dosage. But, as suggested above, some persons, after the 2-drop dose of 0.1% TEPP, noted that they required more intense illumination of the chart in order to achieve the same 20/20 acuity which they had before dosage, whereas, with the test conducted at unvaried illumination intensity, the Ortho-Rater showed a definite loss of acuity at the highest dosage level, averaging about 2 scoring units from 12 to 10 (Snellen equivalent of 20/17 to 20/20 for far vision), for both eyes and for each eye separately at both near and far vision. This slight loss of acuity was most evident during the period (one hour after dosage) when the patients were complaining most of the effects of ciliary spasm, conjunctivitis, rhinitis, and headache. It is quite possible that the chemical irritative effects as well as the diminished light intensity inherent in the miosis may have contributed to this slight loss of acuity.

Depth perception was not changed significantly at any dosage as measured objectively or by subjective complaints.

TABLE 2.—*Ocular Tension in Millimeters of Mercury as Determined by Schiøtz Tonometer Before and After 0.1% TEPP*

Case No.	1 Hr. After Peanut Oil		3 Hr. After TEPP	
	Right	Left	Right	Left
2	19	19	13	13
3	20	17	20	17
4	17	20	16	15
5	25	20	17	15
7	13	13	12	12
9	16	17	15	14
Mean	18.3	17.7	15.5	14.3

Three hours after the administration of 2 drops of 0.1% TEPP, the average ocular tension of the six men dropped 2.8 and 3.4 mm. of mercury in the right and left eyes, respectively, as compared with normal levels established after instillation of peanut oil. The changes are summarized in Table 1 and given in more detail in Table 2. The tension of one man was a high normal after peanut oil and showed a paradoxical rise after only 1 drop of 0.1% TEPP. After the completely separate test with 2 drops of the same concentration, his tension was found to be back to his high normal level, and this same high normal level was still present one month after the last instillation of TEPP.

The averages of the tests for vertical and lateral phoria at near and far vision varied little. However, each of the subjects showed what appeared to be significant shifts in lateral phoria, but not all in the same direction.

Erythrocyte and plasma cholinesterase level, color vision, and perimetry remained normal throughout the entire experiment.

In order to be sure that the effects of the experimentally induced miosis were essentially the same as those of miosis acquired under conditions of actual application of TEPP dust, two pilot volunteers were reexamined during the season of occupational exposure. The rechecks were performed two to two and one-half hours after one pilot applied 2800 lb. of 1% TEPP (plus 1.5% other ethyl phosphates) dust and the other pilot applied 4900 lb. of the same formulation. One pilot showed no notable differences in the test results after the occupationally acquired miosis as compared with results after experimentally induced miosis. The second showed a greater ability to accommodate as measured by negative lenses at the usual Snellen test distance of 20 ft. His ability to read 20/20 was maintained up through − 7.00 D. compared with — 4.50 D. when his miosis was experimentally induced. However, as measured by the Prince Rule, accommodation was less than when miosis was induced. This second pilot also complained of loss of acuity in the right eye only ("has a shade over it" or "things look fuzzy"). The Snellen chart showed no detectable loss of acuity in this right eye, but the Ortho-Rater indicated a drop from his level with induced miosis of 10 to 4 scoring units (Snellen equivalent 20/20 to 20/50) for far vision. The left eye maintained the same acuity of vision after miosis was produced experimentally and occupationally. His acuity for near vision in the right eye dropped even farther after occupational exposure, from a normal of 10 scoring units to 1 unit (Jaeger equivalent: 1 to 10). The reason for this wide difference of results in the two methods of measurement is not yet apparent. It is believed that the repeated loss of acuity in the right eye of this particular pilot indicates a visual defect peculiar to his optical system.

By means of the several studies described thus far, physical explanations were found to account for all the predicted and recognized effects of TEPP in the eye. The one complaint by operating pilots remaining to be explained was their inability to judge distance with self-assurance. Dr. George H. Henton, Ophthalmologist, of Portland, Ore., who reviewed the results of the tests, suggested the possibility that radically unequal accommodation due to the production of miosis in one eye only might produce such effects. This hypothesis was consistent with clinical experience with patients who are placed on unilateral miotic therapy for the first time and also consistent with the infrequency of the inability to judge distance among pilots experiencing miosis after commercial applications. Moreover, several instances of unilateral miosis were discovered among pilots who were recently exposed as well as in old records of exposures.

As a test of this hypothesis, the first volunteer in whom bilateral miosis had been produced was retested with unilateral miosis following instillation of 2 drops of 0.1% TEPP. All ophthalmic results following unilateral induction were essentially the same for the single treated eye as they were for each eye following bilateral treatment. However, this volunteer did feel a little uncertain in going down or climbing stairways for about 12 hours after the instillation.

Six more nonpilot volunteers were given abbreviated testing after induction of unilateral miosis in the left eye. The objective findings were essentially the same (for the treated eye) as were those for the six pilots described earlier, but the volunteers with unilateral miosis had subjective complaints for about 24 hours after the induction. Four of the subjects had no idea what minor disturbances of vision might occur. Because their subjective complaints are not easily subject to classification or quantitation, they are listed here.

CASE 1.—"Indefinable obscuring in the left field of vision and sensation that the two eyes were not on the same plane vertically."

CASE 2.—"The ground is coming up at me." "I feel one-sided." (The morning after induction, this volunteer hit his thumb three times in 20 minutes while attempting to nail fruit boxes. He has done this work for three months before and two months after this incident without such errors of distance judgment.)

CASE 3.—"The pegs are blurred" (on Howard-Dolman, but performance normal). "I feel like I'm stepping farther than I'm actually going. I'm giddy yet this morning." (This patient felt sweaty, especially on the back of his [illegible], about one and one-half hours after the first drop of TEPP. He was slightly nauseated and had tightness in his chest. He was nauseated by odor of ether while passing recovery rooms. This patient had been treated previously for neurocirculatory asthenia.)

CASE 4.—"It feels like it [the eye] is hanging out." "I feel like my left eye is wide open; it is bigger than my right eye and yet it feels like it does no good to look through the left eye." (This man missed the first step when reentering his home after the test.) "On the next afternoon I had the feeling that my left eye stared straight ahead, wide open and saw nothing. However, when I close my right eye, the image in the left looks O.K."

CASE 5.—"It feels cockeyed after the second drop." "I fumbled the sugar bowl, while removing it from a shelf at head height. I almost dropped it and I spilled sugar."

CASE 6.—"I have a vague feeling of not seeing too well." (This volunteer missed a step and almost fell.)

Thus, all seven of the volunteers in whom unilateral miosis was induced complained of subjective inability to perceive distance properly with self-assurance or experienced fumbling, stumbling, or other sensorimotor accidents or both.

Similar complaints frequently occur among ophthalmological patients in whom unilateral mioses is induced for therapeutic reasons. Dr. F. W. Jobe, of the Bausch and Lomb Optical Company, pointed out that these results are doubtless physiologically the same as the Pulfrich effect described by Duke-Elder[8] and in other textbooks. The Pulfrich effect causes the subject with unequal light intensities in the two eyes to perceive moving objects falsely; thus a pendulum appears to rotate in an ellipse instead of in one plane.

The induced or occupationally acquired miosis lasted from 12 to 24 hours, as indicated by the failure of the pupil to react to light and in accommodation. Subjective complaints lasted about the same length of time. Partial constriction of the pupil lasted from two to three days.

Summary and Conclusions

Pilots and others have long recognized the miosis which is frequently a consequence of applying tetraethylpyrophosphate (TEPP) dust by aircraft. Accompanying the miosis, operating pilots have regularly noted a decrease in light perception and have more rarely experienced an inability to judge distance.

Two drops of 0.05% TEPP instilled into each eye of volunteers produced slight miosis and a slight increase of near accommodation.

Two drops of 0.1% TEPP instilled into each eye of volunteers produced maximal miosis, an increase in near and far accommodation, and a decrease in light perception but no inability to judge distance.

Two drops of 0.1% TEPP in only one eye of each of seven volunteers produced unilateral miosis and other expected changes in the treated eye but also caused all the volunteers to complain of difficulty in vision; four of the volunteers complained specifically of difficulty in judging distance, and six of them made sensorimotor errors of fumbling, stumbling, or other clumsiness.

It is concluded that infrequent unilateral contamination of the eye by spillage or other accident, accounts for the occasional inability of pilots applying TEPP to judge distance adequately. The incoordination which may accompany such inability to judge distance may be a serious threat to the pilots.

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Chlorinated Hydrocarbons and Eggshell Changes in Raptorial and Fish-Eating Birds

Abstract. *Catastrophic declines of three raptorial species in the United States have been accompanied by decreases in eggshell thickness that began in 1947, have amounted to 19 percent or more, and were identical to phenomena reported in Britain. In 1967, shell thickness in herring gull eggs from five states decreased with increases in chlorinated hydrocarbon residues.*

New perspectives on the role of chlorinated hydrocarbon insecticides in our environment have come into focus in recent years. Successive discoveries have demonstrated that these compounds are systematically concentrated in the upper trophic layers of animal pyramids (*1*). Raptorial bird populations have simultaneously suffered severe population crashes in the United States and Western Europe (*2, 3, 4*). These involve reproductive failures which, at least in Britain, are characterized by changes in calcium metabolism and by a decrease in eggshell thickness resulting in the parent birds' breaking and eating their own eggs (*4, 5, 6*). Such a derangement of calcium metabolism or mobilization perhaps could result from breakdown of steroids by hepatic microsomal enzymes induced by exposure to low dietary levels of chlorinated hydrocarbons (*7*).

We have examined the possibility that the eggshell changes reported in Britain (*6*) have also occurred in the United States and that the raptor population crashes in Europe and North America may have had a common physiological mechanism. The population changes are without parallel in the recent history of bird populations (*8*). They include the pending extirpation of the peregrine falcon (*Falco peregrinus*) in northwestern Europe, the complete extirpation of the nesting population of this species in the eastern half of the United States, and simultaneous declines among other bird- and fish-eating raptors on both sides of the Atlantic.

We examined 1729 blown eggs in 39 museum and private collections. Shells were weighed to the nearest hundredth of a gram. In 29 percent of these, we were able to insert a micrometer through the hole drilled by the collector at the girth of the shell and to take four measurements of thickness 7 mm from the edge of the blow hole; these were then averaged to the nearest 0.01 mm for each shell. Thickness in each case then represented the shell itself plus the dried egg membranes. Peregrine falcons, bald eagles (*Haliaeetus leucocephalus*), and ospreys (*Pandion haliaetus*) were selected as having one or more regionally declining populations; golden eagles (*Aquila chrysaetos*), red-tailed hawks (*Buteo jamaicensis*), and great horned owls (*Bubo virginianus*) were selected as representative of reasonably stationary populations that may be slowly declining as their habitats are gradually destroyed by man, but for which widespread reproductive failures are currently unknown. In addition, 57 eggs of the herring gull (*Larus argentatus*) were collected from five colonies in 1967. The shells of these were dried at room temperature for 4 months before being measured, and residues of the entire egg contents were analyzed by the Wisconsin Alumni Research Foundation for chlorinated hydrocarbons but not for polychlorinated biphenyls. Analytical procedure followed that outlined by the U.S. Food and Drug Administration (*9*). Analyses were conducted on a gas chromatograph (Barber Coleman, model GC 5000, and Jarrell-Ash, model 28-700) with electron-capture detectors. The glass column (0.6 cm by 1.2 m) was packed with 5 percent DC 200 (12,500) on Cromport XXX. The column temperature was 210°C, and the nitrogen flow rate was 75 cm^3/min. Each portion of the ground and dried samples was extracted for 8 hours or more in a Soxhlet apparatus with a mixture of ether and petroleum ether (70:170). Portions of the extracts were further purified by putting them through a Florisil column.

In California, where the peregrine falcon population is in "a serious condition" (*10*), a change of 18.8 percent in shell weight occurred from 1947 to 1952. Ratcliffe (*6*) found a corresponding decrease of 18.9 percent in Britain. The change in California involved a decrease in shell thickness and had no precedent in the previous 57-year recorded history of the peregrine in that state (Fig. 1). In the eastern United States, where the nesting population of peregrines has now been wiped out (*3*), fragmentary data indicate that the same change took place (Table 1). Broken

Table 1. Weights of raptor eggshells in museum and private collections. Citations (*23–25*) refer to the data for the population trend; S.E., standard error of the mean.

Region	Period	No.	Weight (g)		Population trend (or reproduction)
			Mean ± S.E.	Change (%)	
			Red-tailed hawk		
Calif. (*23*)	1885–1937	386	6.32 ± 0.032		
	1943–44	6	6.09 ± 0.237	− 3.6	Stationary
	1953–67	8	6.49 ± 0.214	+ 2.7	Stationary
			Golden eagle		
Calif. (*23*)	1889–1939	278	13.03 ± 0.083		
	1940–46	28	12.70 ± 0.161	− 2.5	Stationary
	1947–65	33	13.41 ± 0.232	+ 2.9	Stationary
			Bald eagle (24a)		
Brevard Co., Fla.	1886–1939	56	12.15 ± 0.127		
	1947–62	12	9.96 ± 0.280	− 18.0	Declining
Osceola Co., Fla.	1901–44	25	12.32 ± 0.240		
	1959–62	8	9.88 ± 0.140	− 19.8	Declining
			Osprey (24b)		
Md.-Va.	1890–1938	152	7.05 ± 0.054		
	1940–46	21	6.91 ± 0.164	− 2.0	Stationary
	1955	3	6.85	− 2.8	Stationary
N.J.	1880–1938	117	7.08 ± 0.069		
	1957	6	5.30 ± 0.446	− 25.1	Declining
			Peregrine (25)		
B.C.	1915–37		4.24 ± 0.061		
	1947–53	15	4.18 ± 0.081	− 1.4	Stationary
Calif. (*23*)	1895–1939	235	4.20 ± 0.031		
	1940–46	49	4.07 ± 0.038	− 3.1	No data
	1947–52	31	3.41 ± 0.084	− 18.8	Declining
N.H. to N.J.*	1888–1932	56	4.38 ± 0.034		
Vt.	1946	3	4.30	− 1.8	Stationary
Mass.	1947	3	3.47	− 20.8	Extirpated
N.J.	1950	3	3.24	− 26.0	Extirpated
			Great horned owl		
Calif. (*23*)	1886–1936	154	4.50 ± 0.033		
	1948–50	12	4.62 ± 0.119	+ 2.4	Stationary

* Including Vermont and Massachusetts.

3

eggshells in a North American peregrine eyrie were observed for the first time in 1947 by J. A. Hagar 60 miles (9.6 km) from the Massachusetts eyrie cited in this table (*11*). They were next inferred in Quebec in 1948 when egg-eating was observed at the same site in 1949 (*12*), and were observed in Pennsylvania in 1949 and 1950 (*13*). Chlorinated hydrocarbon data for this now-extinct regional population are completely absent. For nine surviving adult peregrines in Canada's Northwest Territories in 1966, the data are reported to have averaged 369 parts per million (ppm) (fresh weight) in fat (*14*). For four adults in another migratory population in northern Alaska, values were even higher (*15*).

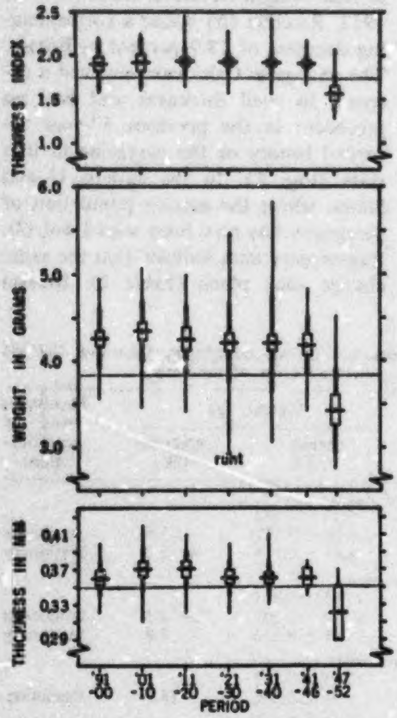

Fig. 1. Measurements of 614 California peregrine eggshells collected since 1891. The dotted horizontal line is the midpoint between the 95 percent confidence limits for 1947–52 and the lowest of any preceding group. Solid horizontal bars are means; rectangles, 95 percent confidence limits; heavy vertical lines, standard deviations; narrow vertical lines, range in sample. The thickness index, calculated as ten times the weight divided by the product of the length and breadth (in mm) of each egg, was devised by Ratcliffe (*6*) for the study of museum eggs and appears here to be a meaningful statistic. The sizes of samples for measurement of weight and of thickness index for the periods were 71, 49, 36, 85, 155, 30, and 31, respectively; and the sizes of the samples for measurement of thickness were 24, 31, 29, 23, 37, 7, and 6.

For the five other raptorial species we have studied, the data do not permit a precise delineation of the onset of the change in calcium metabolism or mobilization, but the decrease (Table 1) in shell weight (and hence thickness) has involved only declining populations and not stationary ones. Change in shell thickness occurs in poultry as a result of dietary deficiencies and age (*16*, *17*). This phenomenon would probably not occur simultaneously on two continents 1 year after the chlorinated hydrocarbon insecticides came into general usage. Other chemicals affect shell thickness in poultry (*17*), but the finding of high concentrations of chlorinated hydrocarbons in the eggs of wild populations of raptors and the time correlation of shell changes with the introduction of DDT [1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane] tend strongly to suggest that chlorinated hydrocarbons are the major contributing cause, although it is not unlikely that other chemicals could be contributory.

In order to test the hypothesis that these recent changes of thickness in raptor eggshells were the result of differences in exposure to chlorinated hydrocarbons we analyzed 10 to 14 eggs taken in 1967 from each of five colonies of the herring gull (*Larus argentatus*). Mean shell weight and thickness in 55 eggs collected in the same five states prior to 1947 disclosed no geographic gradients or significant differences. The 1967 mean thicknesses for each colony were therefore compared to mean levels of residual DDE [1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene] on a fresh-weight basis, with the result shown in Fig. 2, the *r* value being significant, with $P = .001$. The residues of polychlorinated biphenyls (*18*) have not been studied in these ecosystems, but DDE has been consistently high in the Lake Michigan birds, averaging (fresh weight) 1925 ppm (S.E. 274) in the fat of 12 healthy adults collected in 1963–64 (*19*).

Reproduction in these gull colonies was generally normal in 1967 except perhaps in Wisconsin. At the latter colony where an 11 percent mean decrease in shell thickness occurred, some egg breakage and shell flaking was evident in 1967, although not at the frequency seen in previous years. Excessive reproductive failure occurred at this site in 1964 when about 18 percent of the eggs lost about one-third of the shell due to flaking, when clutch size decreased and embryonic mortality was high, and when DDE residues averaged 202 ppm (S.E. 34) in nine eggs (*20*). (If linear extrapolation of the 1967 values is carried out to 202 ppm, the shell thickness in 1964 could be estimated as having decreased by about 32 percent.) The effectiveness of DDE in the enzymatic metabolism of aminopyrine has been reported by Hart and Fouts (*21*), and our data suggest that this compound, because of its prevalence, has played a major role in inducing the hepatic microsomal metabolism of steroids that in turn resulted in the eggshell changes we have encountered in museum collections. Without doubt DDE is the commonest insecticide or insecticide analog now being found in avian tissues (*22*). In 1966, it was found to average 284 ppm (S.E. 62) in the fat of nine arctic-breeding peregrine falcons (*14*) and about 414 ppm in four others on a wet-weight basis (*15*). Concentrations of this compound and other chlorinated hydrocarbons in the peregrine populations that crashed farther south can be assumed to have been as high—and they may have been much higher.

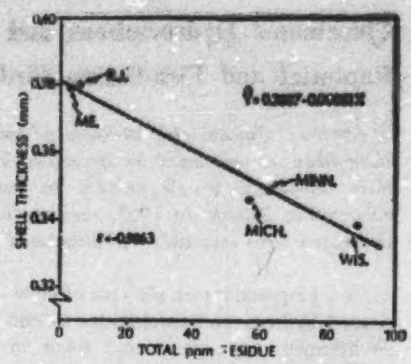

Fig. 2. Variation in shell thickness and DDE concentrations in the eggs of herring gulls in 1967. The eggs were taken off Block Island, R.I.; Green Island in Penobscot Bay, Me.; Rogers City, Mich., on Lake Huron; near Knife River, Minn., on Lake Superior; and the Sister Islands in Green Bay, Wis. Some polychlorinated biphenyls probably occurred in these eggs, but they have not yet been identified.

From the above evidence and that accumulated by others (*2*, *4*, *6*, *8*), we have reached these conclusions: (i) many of the recent and spectacular raptor population crashes in both the United States and Western Europe have had a common physiological basis; (ii) eggshell breakage has been widespread but largely overlooked in North America; (iii) significant decreases in shell thickness and weight are characteristic of the unprecedented reproductive failures of raptor populations in certain parts of the United States; (iv) the onset of the calcium change 1 year after the introduction of chlorinated hydrocarbons into general usage was not a random circumstance; and (v) these persisting compounds are having a serious insidious effect on certain species of birds at the tops of contaminated ecosystems.

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